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Veterinarians Dedicated to Animal Reproduction

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The purpose of *Clinical Theriogenology* is to publish in a timely manner peer-reviewed information relevant to the clinical practice of theriogenology for veterinary practitioners, academic clinicians, and veterinary students. The journal will be the means by which the Society for Theriogenology (SFT) publishes the proceedings of its Annual Conference and Symposia.

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Clinical Theriogenology will be broad in scope and manuscripts published will be in the following categories:

- Research reports
- Reviews of current literature
- Clinical reports
- Innovative techniques
- Book reviews
- Letters to the editor
- Editorial opinion
- News from the Society for Theriogenology and the American College of Theriogenologists

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Clinical Theriogenology

Contents

September 2014, Volume 6, Number 3

David E. Bartlett Award for Lifetime Achievement in Theriogenology
A LIGHTER SIDE OF THERIOGENOLOGY
Bob BonDurant 135

DAVID E. BARTLETT 1917-2014
Paul Bartlett 139

Society for Theriogenology 60th Anniversary
THE SOCIETY FOR THERIOGENOLOGY CELEBRATES 60 YEARS
R. S. Youngquist 141

General Session
ENHANCING THE LEARNING EXPERIENCE OF STUDENTS IN REPRODUCTIVE SCIENCE WITH MULTIMEDIA PLATFORMS
A.C. Oki, J.G. Berardinelli, J.A. Clapper, D.H. Poole, P.L. Senger 147

Small Animal Session
RECENT ADVANCES IN SWINE REPRODUCTION
Sherrie G. Clark 155

MINIATURE PET PIG REPRODUCTION
Sherrie G. Clark 165

COMMON PRACTICES IN MANAGEMENT OF BREEDING DOGS AND PUPPIES: A SURVEY OF 461 DOG BREEDERS
Margaret V. Root Kustritz 171

IMAGING OF THE SMALL ANIMAL FEMALE REPRODUCTIVE SYSTEM
Judith A. Hudson 189

IMAGING OF THE SMALL ANIMAL MALE REPRODUCTIVE SYSTEM
Judith A. Hudson 195

WINDOWS OF OPPORTUNITY: A NEW LOOK AT CANINE SOCIALIZATION
Christopher L. Pachel 201

REPRODUCTIVE DISORDERS OF THE SPAYED BITCH
Cheryl Lopate 205

EVALUATION OF THE TOM: COLLECTION PROCEDURES, EVALUATION OF SPERM, AND SUBSEQUENT USE
Aime K. Johnson 219

EVALUATION AND INSEMINATION OPTIONS FOR THE SUBFERTILE QUEEN
Aime K. Johnson 225

EFFECT OF ANTIBACTERIAL AGENTS IN SEMEN EXTENDER ON BACTERIAL GROWTH IN EXTENDED CANINE SEMEN HELD AT 5°C AND 20°C FOR UP TO 48 HOURS
Carla Barstow, Margaret V. Root Kustritz 231

Production Animal Session
SAFETY AND EFFICACY OF VIRAL VACCINES FOR BOVINE REPRODUCTIVE PATHOGENS
M. Daniel Givens 239

UPDATE ON TESTICULAR INFECTIONS WITH BOVINE VIRAL DIARRHEA VIRUS
Benjamin W. Newcomer, M. Daniel Givens 243

EPIZOOTIC BOVINE ABORTION (FOOTHILL ABORTION): THE DISEASE, DIAGNOSIS AND CONTROL STRATEGIES
Jeffery Scott 247

CHLAMYDIA AND CHLAMYDOPHILIA IN BOVINE REPRODUCTION
Johannes Kauffold, Axel Wehrend, Haukur Sigmarsson 251

CHLAMYDIA AND CHLAMYDOPHILIA IN SMALL RUMINANTS AND OTHER FARM ANIMALS
Johannes Kauffold, Axel Wehrend, Haukur Sigmarsson, Matthias Hoops 255

SCHMALLENBERG VIRUS IN EUROPE-A REVIEW
Johannes Kauffold, Thomas W. Vahlenkamp, Matthias Hoops 261

BOVINE ABORTION: DIAGNOSTIC METHODS
Larry D. Holler 267

OVINE INFECTIOUS ABORTION
Larry D. Holler 271

REDISCOVERING THE SILENT ENEMY IN CATTLE REPRODUCTION HEALTH: FROM THE LATEST FINDINGS ABOUT SEXUALLY TRANSMITTED TRICHOMONIASIS AND CAMPYLOBACTERIOSIS TO THE CURRENT CONTROL AND FUTURE THERAPEUTICS
Eduardo R. Cobo, Pedro H. Favetto 277

Equine Session
APPLICATION OF EQUINE OOCYTE RECOVERY AND ASSISTED REPRODUCTIVE TECHNIQUES TO CLINICAL PRACTICE
Rob Foss 293

THE EQUINE EMBRYO ADVANCES IN TECHNIQUES: TROPHECTODERM BIOPSY AND EMBRYO VITRIFICATION
Elizabeth Metcalf 297

IMMUNOLOGY AND IMMUNOMODULATION OF THE REPRODUCTIVE TRACT
Kristina Lu, Peter Morresey 303

EQUINE SUBCLINICAL ENDOMETRITIS CAUSED BY DORMANT BETA-HEMOLYTIC STREPTOCOCCI
M.R. Petersen, A.M. Bojesen 313

BACTERIAL ENDOMETRITIS: A FOCUS ON BIOFILMS
Ryan A. Ferris 315

EQUINE VIRAL ARTERITIS – AN UPDATE
G. Reed Holyoak, Candace C. Lyman, Udeni B. R. Balasuriya 321

USE OF MOLECULAR DIAGNOSTICS FOR INFECTIOUS EQUINE PATHOGENS
Nicola Pusterla 329

BIOSECURITY APPROACHES FOR EQUINE REPRODUCTION
Nicola Pusterla 335

Opening Session Abstracts
BILATERAL ANORCHIA IN A DOG
Justin W. McNaughten, Audrey A. Kelleman 339

DIAGNOSIS AND MANAGEMENT OF A GRANULOSA-THECA CELL TUMOR IN A NULLIPAROUS HOLSTEIN HEIFER
J.N. Roberts, C.L. Carleton, D.W. Agnew 340

SEMEN COLLECTION FROM A STALLION WITH A PERMANENT PERINEAL URETHROSTOMY: A CASE REPORT
Patrick M. McCue, James Speer, Paula Moffett, Kristen Loncar, Chelsie Burden, Ryan A. Ferris 341

THE CANINE CORPUS LUTEUM EXPRESSES IGF1 AND ITS RECEPTOR: IMPLICATIONS IN LUTEAL FUNCTION
O. Balogh, A. Boos, M.P. Kowalewski, I.M. Reichler 342

ACCURACY OF BEHAVIORAL TESTING FOR PREGNANCY DIAGNOSIS IN THE DROMEDARY CAMEL
A. Tibary, J.S. Rodriguez, L.K. Pearson, A. Campbell 343

NEONATAL EXPOSURE TO MEDROXYPROGESTERONE ACETATE ALTERS CANINE UTERINE DEVELOPMENT
Robyn R. Wilborn, Anne A. Wiley, Katharyn I. Brennan, Aime K. Johnson, Natalie S. Fraser, Ghislaine A. Dujovne, Paul S. Cooke, Frank F. Bartol 344

PRENATAL EXPOSURE TO AN ANDROGEN AGONIST AND ANTAGONIST ALTERS THEOVINE SEXUALLY DIMORPHIC NUCLEUS IN MALE BUT NOT FEMALE LAMB FETUSES
C.T. Estill, M. Meaker, F. Stormshak, H.J. Montilla, C.E. Roselli 345

LEUKOCYTE ESTERASE TEST FOR DIAGNOSIS OF ENDOMETRITIS IN DAIRY COWS
Nicolas Tison, Émile Bouchard, Rejean Lefebvre 346

Rains Memorial Abstract Competition
VISCOELASTIC MODIFIERS AND HYPERACTIVATING AGENTS AS POTENTIAL SELECTORS OF SUPERIOR SPERM FOR *IN VITRO* FERTILIZATION
S.M. Lawlis, S.H. Cheong 347

EVALUATION OF THE EFFECTS OF PRE-BREEDING BEEF HEIFER MANAGEMENT ON MID TO LATE GESTATION UTEROPLACENTAL HEMODYNAMICS
A.J. Cain, C.O. Lemley, F.K. Walters, D.L. Christiansen, E.H. King, R.M. Hopper 348

EFFECTS OF DIFFERENT APPLICATIONS OF PYRETHRIN/PYRETHROID INSECTICIDES ON BULL REPRODUCTIVE PARAMETERS
J.L. Stewart, F.A. Ireland, T.L. Felix, D.W. Shike, V.L. Jarrell, C.F. Shipley 349

COMPARISON OF SURGICAL VASECTOMY OR CHEMICAL EPIDIDECTOMY AS A STERILIZING METHOD FOR FERAL HORSE POPULATIONS
C.M. Scully, R.L. Lee, L. Pielstick, G. Collins, M.A. Kutzler 350

CAN SERUM AMYLOID A PREDICT REPRODUCTIVE EFFICIENCY IN THE POSTPARTUM OF MARES?
Derek G. Howell, Charles F. Scoggin, Marco A. Coutinho da Silva 351

THE EFFECTS OF BOVINE SPERM-BOUND ANTI-SPERM ANTIBODIES ON CAPACITATION
Jessica Klabnik-Bradford, Lynda Miller, Catherine L. Ewen, Maria Soledad Ferrer 352

ENDOMETRIAL TISSUE CONCENTRATIONS OF CEFTIOFUR FOLLOWING INTRAUTERINE INFUSION IN MARES
C.J. Scott, B.W. Christensen, G.A. Dujovne 353

RELATIVE SPERM VOLUME SHIFT, AQUAPORIN 7 MRNA ABUNDANCE AND BULL FERTILITY ESTIMATES
R. Shutter, V. Kasimanickam, R. Kasimanickam 354

Student Case Presentations
MANAGEMENT OF ANEJACULATION IN A STALLION ASSOCIATED WITH NEUROLOGIC DEFICITS
R.E. McPhail, A. Kiser, C.C. Love 355

TRANSRECTAL CRANIO-CERVICAL DISLOCATION OF A TWIN FETUS IN A MARE
E. Putt, B.W. Christensen, A. Wimmer 356

HYDROALLANTOIS AND HYDROAMNIOS ASSOCIATED WITH A BULLDOG CALF AND ABNORMAL PLACENTATION IN A MINIATURE SCOTTISH HIGHLANDER COW
Sarah Woods, Bret McNabb, Catalina Cabrera 357

SEGMENTAL APLASIA OF THE TUBULAR REPRODUCTIVE TRACT IN A SHORTHORN HEIFER
Dana A. Schechter, Marco A. Coutinho da Silva 358

BREEDING MANAGEMENT IN A REINDEER (*RANGIFER TARANDUS*) WITH A HISTORY OF REPRODUCTION FAILURE
A.N. Fadden, N.I. Bott, M.A. Kutzler 359

UNILATERAL UTERINE PROLAPSE IN A 7-YEAR OLD MULTIPAROUS CAT
Christine Garrett, Aime Johnson 360

Species Abstracts
Small Animals
COMPARISON BETWEEN IMMUNOCASTRATION AND SURGICAL CASTRATION IN DOGS
Ajadi Temitope Ayisat; Oyeyemi Mathew Olugbenga 361

COMPARISON OF PENILE SPINES AND SPERM MORPHOLOGY BETWEEN JUVENILE AND ADULT FERAL TOM CATS
Ellie Bohrer, Anna Mihalyo, Michelle Kutzler 362

EVALUATION OF PSEUDOPREGNANCY IN THE AMERICAN BLACK BEAR (*URSUS AMERICANUS*)
Sherrie Clark-Deener, J. Bernardo Mesa, Tess Pangle, Marcella J. Kelly 363

SURGICAL CORRECTION OF A PERINEAL FISTULA IN A 9 MONTH OLD FEMALE DANDIE DINMONT TERRIER WITH URINARY INCONTINENCE
C. Scully, M. Milovancev, H. Montilla, S. Nemanic, C. Estill 364

POST-NATAL EXPRESSION OF CYTOCHROME P450, FAMILY 26 GENES IN CANINE TESTIS
Seth Bynum, Vanmathy Kasimanickam, Ramanathan Kasimanickam 365

DEVELOPING A NON-SURGICAL CONTRACEPTIVE METHOD FOR FEMALE DOGS
A.M.S. Papu-John, T.M. Prado, K.A. Fecteau, R.L. Donnell, S.M.L.C Mendis-Handagama 366

COMPARISON OF CANINE SPERMATOZOAL RNA CONCENTRATIONS AND PURITY USING TWO DENSITY GRADIENT CENTRIFUGATION SOLUTIONS
R.M. Hegedus, C.E. Donovan, A.R. Menino, M.A. Kutzler 367

Production Animals
COMPARISON OF TWO ELECTROEJACULATION METHODS IN RAMS
C.M. Scully, H.J. Montilla , C.T. Estill 368

FATTY ACID COMPOSITION OF THE YOLK SAC MEMBRANE IN THE CHICK EMBRYO
Persia Neumann, Francine Vercese, Gita Cherian, Michelle Kutzler 369

CYSTIC ENDOMETRIAL HYPERPLASIA WITH HYDROMETRA AND INAPPROPRIATE LACTATION IN A NIGERIAN DWARF GOAT, A CASE REPORT
Heidi Fishman, Robert Stawicki, Roberto Palomares 370

COMPARISON OF TWO SUPEROVULATION PROTOCOLS IN THE DROMEDARY CAMEL (*CAMELUS DROMEDARIUS*)
J.S. Rodriguez, L.K. Pearson, A.J. Campbell, M. Ciccarelli, A. Tibary 371

EVALUATION OF A MONDAY-FRIDAY 4-DAY CIDR + TIMED ARTIFICIAL INSEMINATION PROTOCOL IN DAIRY HEIFERS: A PILOT STUDY
Roberto A. Palomares, Joseph Haslett, Karissa Carpenter, Juan Gutierrez, Emmanuel Rollin, Lee Jones, Fernando Perea 372

EFFECT OF 14 D CIDR TREATMENT PRIOR TO 7 D CIDR CO-SYNCH
Sherrie Clark-Deener, Sarah Holland, John Currin, Hollie Schramm, Dee Whittier 373

EVALUATION OF VAGINOSCOPY IN EWES UTILIZING THE SILS™ PORT
Desiree R. Shasa, Jeremiah T. Easley, Jennifer N. Hatzel 374

COMPARISON OF TWO SEMEN EXTENDERS FOR CRYOPRESERVATION OF BIGHORN SHEEP (*OVIS CANADENSIS CANADENSIS*) EPIDIDYMAL SPERM
A.J. Campbell, L.K. Pearson, M. Ciccarelli, A. Tibary 375

Equine

COMPARISON OF SERUM AMYLOID A CONCENTRATIONS IN THOROUGHBRED MARES EXPERIENCING EMBRYONIC LOSS AND IN MARES MAINTAINING PREGNANCY
M.R. Schnobrich, C. Howard, E.A. Bradecamp, W.T. Riddle, B.K. Barber, C. Cray, M.H.T. Troedsson, E. L. Squires, B.A. Ball 376

NECROSIS OF THE SPERMATIC CORD, EPIDIDYMIS, AND TESTIS OF A FRIESIAN STALLION CAUSED BY *SALMONELLA ABORTUS EQUI*
A.P. Bouchard, T.M. Prado, J. Schumacher, R.L. Donnell 377

FOLLICULAR DYNAMICS FOLLOWING CLOPROSTENOL ADMINISTRATION IN THE DIESTROUS MARE
Chelsie A. Burden, Ryan A. Ferris, Patrick M. McCue 378

EFFECT OF EARLY MEDIUM PH ON EQUINE EMBRYO DEVELOPMENT IN VITRO AFTER INTRACYTOPLASMIC SPERM INJECTION
Y.H. Choi, I.C. Velez, B. Macías-García, K. Hinrichs 379

SPERM MOTILITY AND FERTILITY OF COOLED PRESERVED STALLION SEMEN IN EITHER INRA96 OR EQUIPRO COOLGUARD EXTENDER
C.E. Fedorka, G.M. Davolli, B.A. Ball, E.L. Squires, M.H.T. Troedsson 380

URETHRAL DIVERTICULUM IN A BREEDING STALLION
M. Diel de Amorim, A. Rauch, C. Card 381

INCIDENCE RATE AND EFFECTS OF PERSISTENT MATING-INDUCED ENDOMETRITIS (PMIE) IN QUARTER HORSE MARES
Katherine G. DeHaan, Ryan A. Ferris, Patrick M. McCue 382

DEHYDROEPIANDROSTERONE SULFATE AND TESTOSTERONE CONCENTRATIONS IN MARES CARRYING NORMAL PREGNANCIES
Igor F. Canisso, Barry A. Ball, Alejandro Esteller-Vico, Edward L. Squires, Mats H. Troedsson 383

Large Animals

THE INFLUENCE OF THE HORMONAL ENVIRONMENT AND A BACTERIAL CHALLENGE ON GLOBAL GENE EXPRESSION IN THE EQUINE UTERUS
C.D. Marth, N.D. Young, L.Y. Glenton, G.F. Browning, D.M. Noden, N. Krekeler.............................. 384

EFFECT OF FROZEN-THAWED GRANULOSA CELLS AND SERUM ADDITIVES ON INDIVIDUAL BOVINE EMBRYO DEVELOPMENT
Bethany Gibson, Alan Ealy, Willard Eyestone, Sherrie Clark-Deener 385

FERTILITY IN GOATS DURING THE NON-BREEDING SEASON USING A SHORT-TERM PROGESTERONE PRIMING PROTOCOL WITH EITHER GNRH OR HCG TO INDUCE OVULATION
Sandra L. Ayres, Kelly L. Chevett 386

REPRODUCTIVE PERFORMANCE FOLLOWING LONG- VS SHORT-TERM PROGESTERONE-BASED SYNCHRONIZATION PROTOCOLS FOR FIXED-TIME ARTIFICIAL INSEMINATION IN BEEF HEIFERS
S. Schroeder, R. Kasimanickam, J.B. Hall, W.D. Whittier 387

EVALUATION OF EFFECTS FROM CHLORHEXIDINE HYDROCHLORIDE INTRAUTERINE SUSPENSION ADMINISTRATION IN NORMAL MARES
Natalie S. Fraser, Aime K. Johnson, Robyn R. Wilborn, Ghislaine A. Dujovne, Leland Nuehring 388

A SPLIT-DOUBLE INTRAMUSCULAR ADMINISTRATION OF FOLLTROPIN®-V WITH HYALURONAN - A NEW APPROACH TO SUPEROVULATION FOR EMBRYO FLUSHING IN EWES
T.T. Thompson, A. Thompson, A. T. Peter 389

DEEP-HORN INTRA-UTERINE APPLICATION OF PROSTAGLANDIN E_2 DOES NOT HASTEN OVIDUCTAL TRANSPORT OF EQUINE EMBRYOS
M.R. Schnobrich, D.K. Vanderwall, E.A. Bradecamp, D.K. Price, R.D. Stott 390

COMPARISON OF CAUDA EPIDIDYMAL SPERM MORPHOLOGY FOLLOWING SURGICAL VASECTOMY OR CHEMICAL EPIDIDECTOMY IN FERAL HORSES
C.M. Scully, R.L. Lee, L. Pielstick, G. Collins, M.A. Kutzler 391

Posters

UTERINE ARTERY BLOOD FLOW AND ARTERIAL DIAMETER CORRELATE CLOSELY WITH GESTATIONAL AGE IN THE MARE
T.M. Beachler, C.S. Bailey, R.B. Sper, C.N. Buchanan, E.H. Griffith, L. Borst, K.A. McKelvey 392

DIAGNOSTIC DOUBLE GUARDED LOW-VOLUME UTERINE LAVAGE IN MARES
M. Christoffersen, L. Brandis, J. Samuelsson, A.M. Bojesen, M.H.T. Troedsson, M.R. Petersen 393

EFFECTS OF A THIRD-GENERATION GNRH ANTAGONIST ON REPRODUCTIVE PARAMETERS IN THE STALLION
G. M. Davolli, B. A. Ball, A. Esteller-Vico, A. Claes, I. Canisso, C. Fedorka, E. Woodward, M. H. Troedsson, E. L. Squires 394

SUDDEN INFERTILITY OF A STALLION COLLECTED WITH A COMMERCIAL ARTIFICIAL VAGINA
A.P. Bouchard, T.M. Prado, C.R. Pinto, S.S. Hayden, B.K Whitlock 395

COMPARISON OF TWO PHARMACOLOGIC PROTOCOLS TO INDUCE EX COPULA EJACULATION IN NOVICE STALLIONS
L.K. Pearson, E. Larsonberg, A.J. Campbell, A. Tibary 396

LONGEVITY OF CHILLED CANINE SEMEN WITH FRESH EXPRESS®
M. Casal, L. Brody, S. Gotwals, M. Krecic 397

EVALUATION OF ETONOGESTREL, ALTRENOGEST AND MEDROXYPROGESTERONE BIOACTIVITY IN MARES USING AN IN VITRO BIOASSAY
Ghislaine Dujovne, Alan Conley 398

THYROID HORMONE PROFILES IN PREGNANT EUTHYROID AND SUPPLEMENTED HYPOTHYROID BITCHES
J.T. Cecere, D.L. Panciera, B.J. Purswell 399

EFFECTS OF TRANSVAGINAL OVARIAN BIOPSY ON HEALTH AND FERTILITY IN MARES
Dawne Nairn, Stephen Manning, Ilse Dedden, Elinorah Ripley, Claire Card 400

EFFECTS OF LOW SERUM PROGESTERONE CONCENTRATIONS ON ENDOMETRIAL TRANSCRIPTION AT DAYS 8 AND 12 OF THE ESTROUS CYCLE IN MARES
A. Esteller-Vico, K.E. Scoggin, E.L. Squires, M.H. Troedsson, B.A. Ball 401

TRANSCERVICAL ENDOSCOPIC CATHETERIZATION TECHNIQUE WITH UTERINE LAVAGE TO IMPROVE CLINICAL OUTCOMES OF MEDICALLY MANAGED PYOMETRA IN THE BITCH
Marthina Greer, J. Curtis Zella, Cheryl Lopate, John Verstegen 402

A COMPARATIVE EFFICACY TRIAL FOR TIMETHOPRIM-SULFAMETHOXAZOLE, GENTAMICIN AND PENICILLIN, USING AN *EX VIVO* MODEL OF GESTATIONAL DISEASE
Katherine McKelvey, Megan Jacob, Mark Papich, Theresa Beachler, C. Scott Bailey 403

ENDOCRINE AND MOLECULAR CHANGES IN THE EQUINE FOLLICLE ASSOCIATED WITH AGEING IN MARES
A. Claes, B.A. Ball, K.E. Scoggin, C.E. Fedorka, E.L. Squires, M.H.T Troedsson 404

Student Case Posters
TESTICULAR ABSCESS IN AN ABERDEEN ANGUS BULL
Jessica Klabnik-Bradford, Maria Soledad Ferrer, Matt Miesner, Gretchen Grissett, Jessica Monday, Lynda Miller 405

UNUSUAL SYSTEMIC RESPONSE TO ARTIFICIAL INSEMINATION IN A MAIDEN MARE
D. Andrew Hestad, Robyn Wilborn, Aime Johnson 406

METASTATIC NEOPLASIA IN A POSTPARTUM HOLSTEIN HEIFER
S.E. Schroeder, A.J. Campbell, J.F. Muñoz-Gutiérrez, L.K. Pearson 407

Manuscripts from pre-conference symposia will be included in subsequent issues of *Clinical Theriogenology*.

The 2014 Bartlett Address
A lighter side of theriogenology

Bob BonDurant
Professor Emeritus, School of Veterinary Medicine, University of California-Davis, Davis, CA

It is with sincere humility that I accept the David E. Bartlett award for 2014. My respect for those who have won this award in years past causes me to beg the question: *What was the nominating committee thinking this year?* After all, I was a bureaucrat for much of my career, a result of a character weakness that made it difficult to form and deliver the word "no".

A very brief and mostly accurate bit of history

It is customary at this presentation to list a bit of history of the SFT/ACT. First, there is no truth to the rumor that the Society selected "Theriogenology" as its moniker because it couldn't fit "Obstetrics, Gynecology & Andrology" on a business card. Rather, the Society grew out of a pioneer group of veterinary clinicians and scientists who called themselves "The Rocky Mountain Society for the Study of Breeding Soundness in Bulls," later changed to the "American Society for the Study of Breeding Soundness." So the Society has a long history of loquacious titles.

Through the efforts of Dr. David Bartlett and others, notably Dr. Herbert Howe, Professor of Classics at the University of Wisconsin, a definitive name was chosen: The Society for Theriogenology (therio: beast; gen: origin of; and ology: the study of), accurately reflecting the subject matter and activity of veterinarians with an interest in reproduction "of beasts." Later, a veterinary specialty encompassing the broader topic of reproductive physiology, reproductive pathology, and reproductive medicine and surgery in farm animals, pets, and wildlife, was recognized by the AVMA as the American College of Theriogenologists. So the ACT was formed as a specialty field of scientific/clinical activity, and the SFT was designed as an educational/teaching organization.

Actually, the name of the ACT has attracted a great deal of discussion, inasmuch as it was intended to be inclusive of basic and clinical fields of endeavor; male and female reproductive biology; and all species of mammals (and occasionally beyond mammals). It is especially pleasing that this goal of large and small animal theriogenologists has gained stature with the initiation of new residencies in small animal theriogenology at Pennsylvania and Auburn and at my alma mater, UC-Davis. Some of these positions are industry-funded, an increasingly used route to support full time graduate clinical studies (residencies).

I'm honored beyond description to receive this David E. Bartlett Award, not only because it comes from my colleagues, but also because it bears the name of one with whom I shared a particular interest, namely trichomoniasis in cattle.

People who have influenced me

I would like to recognize a few of the people upon whose shoulders I and many others stood. There are more than I can list here, but I want to acknowledge these. Not all are card-carrying theriogenologists, and not everyone was a warm and fuzzy type, but all have influenced me in a very positive way:

John Kendrick - One of the early members of the SFT, and an editor of *Theriogenology*. He broke me in to academic citizenship, and helped me survive academia. His untimely death was a personal loss for me.

John McCormack - He taught me the value of working *for the client*, maintaining one's integrity, and the value of a thorough physical examination. He also taught me the value of a sense of humor, and his is especially delightful.

George Stabenfeldt - He mentored me through the preparation of several papers, lectures, and seminars. He also taught me the value of a golf break now and then. He died much too young.

John Hughes - John was a gentleman, to be sure, but he and I shared a difficulty in remembering people's names, especially when our wives were not standing close by to discreetly remind us of an

approaching colleague's name. Well, I had just been hired and John was my chairman, and for some reason he called me Ron. This was in the hippie-dippie days of the late 1970's, and I wasn't sure that John approved of my three-layers of embroidered denim work shirts, or my longish hair. Anyhow, I passed Dr. Hughes in the hall of the teaching hospital one day and 15 feet down the hall, he turned and called out "Ron, could I see you in my office?" Uh-oh, I thought, I'm not even through my probationary period and he's going to fire me. He motioned me into his office, had me sit, and slid open a drawer, revealing three neatly pressed and folded dress shirts. "Ron", he said, "If I give you these shirts, will you wear them?" I knew my leverage would never be greater, so I said "Dr. Hughes" (I always called him Dr. Hughes), "If you'll call me Bob, I'll wear the shirts." He did and I did, and we were friends thereafter.

Al Conley - A true comparative theriogenologist, an exacting scientist, and my go-to guy for physiological and endocrinological updates (anything after about 1978); he is also a source of much intellectual stimulation. He puts the fun in fundamental research.

Carlos Campero - In the 1990's, this DVM, Ph.D, a gentle Argentine soul, came to our laboratory via a fellowship. Carlos' sweet demeanor belied his Herculean work ethic; he was a collaborator on the histopathology of experimental trichomoniasis. He taught me the value of maintaining a family life along with one's busy professional life.

Mark Anderson - A pathologist for the California Animal Health and Food Safety Laboratory, was part of the team that participated in both publicly and privately funded work on the pathogenesis of bovine trichomoniasis. His cheerful enthusiasm for the projects was infectious.

Lynette Corbeil - Known to many of you. She has perhaps done as much for theriogenology of livestock as any non-member of the Society. She is a UC San Diego (and UC Davis) veterinary microbiologist whom I met over the phone. It was the most serendipitous phone call I ever made. I called her to see if our little journal club was interpreting her results concerning female reproductive tract immunity as she did, and further [gulp], would she want to collaborate on trichomoniasis research? She graciously said "Yes," and the collaboration lasted for more than 20 years.

Several years ago the NIH hosted a symposium at the Hamilton laboratory in Montana. The title/subject of the meeting was: "Prospects for Molecular Vaccines for [non HIV] Sexually Transmitted Diseases." (Was it named by the same committee that named the SFT?) There were two veterinarians in attendance: Lynette Corbeil and myself. The rest of the high-powered types at the meeting got up and talked about the biology of their favorite non HIV STD bug, concluding with the phrase "and that's why there will probably never be a vaccine for X.". From the back of the room came a small stirring and a righteous Dr. Corbeil advanced to the front of the room where the microphone was. "I don't know what all this gloom and doom is about STD vaccines," she said. "We've had a relatively efficacious and commercially available vaccine for bovine venereal campylobacteriosis for years." One could have heard a pin drop. Also, it was at this conference that I heard the term "herd immunity" applied to the human species for the first time. It wasn't especially new, but it sounded like they had discovered it.

Maarten Drost - Whatever precision I have adopted, I have adopted it from Maarten. He has been a mentor, a friend, and a collaborator throughout my career. It was a fitting finish to my career that I ended it as I began it, behind a cow (in this case a water buffalo), with Maarten.

Linda Munson - There are many stories I could tell about Linda, mostly dealing with her strength and determination and her courage. I choose to remember her as proud and funny, such as the time when I suggested that, in order to save time on a collaborative project we were working on, I might read the histopathology slides instead of Linda; to which she replied, only with a gesture, i.e., she dropped her glasses down her nose and stared at me. I got the point

Gary Anderson - Introduced to me by Maarten Drost, Gary, who was in the Department of Animal Sciences, UC Davis became a life-long friend. His sparkling wit and appetite for hard work - physical and mental - were an inspiration. As the captain of the Embryo Transfer team he directed the efforts of Animal Science faculty and graduate students, veterinary faculty and students, and technical staff. And he patiently taught basic embryology to a rookie vet school faculty member. He retired to Michigan, but not before leaving us with a lifetime's worth of stories.

Peter Kennedy - A treasure of knowledge and anecdotes, and a killer tennis player. I was lucky enough to coauthor a paper with him and a couple of other faculty members. Peter was on sabbatical leave in Australia, out of immediate reach, i.e., before the internet, email, etc. Between the time the paper was submitted and the time it was published, one of the co-authors died. I duly noted that status change with an asterisk and an explanation, sent out copies of the proofs to all co-authors, and returned the paper to the journal for publication, with instructions to send Dr. Kennedy some copies of the final published paper. Apparently, the asterisk caught his attention, because it was squarely over *his* name, instead of being over the deceased's name. His faxed response simply said "Hey, I'm down under, but I'm not down under *there*."

Stupid things I wish I'd never said or done

The opportunity to collaborate with the above people and many others during my career has been a privilege. But not all collaborations went so well. I still cringe with shame and horror at the time that I stuck my foot in it while collaborating with a Human Infertility Clinic. They were working on a sperm-in-mucus assay, using the bovine as a model. One afternoon, I brought bovine semen and cervical mucus samples down to the laboratory, as was my habit on Wednesdays. Dr. Sikes (not his real name) didn't look up from his microscope, but motioned me to put the samples on the bench. I did so, and went down to the locker room to change out of my coveralls. When I came back to the laboratory, Dr. Sikes was still looking through the 'scope, but he had the TV monitors on, and an undiluted, unstained semen sample with a few spermatozoa struggling across the field displayed <u>on</u> those monitors. Many of those sperm that were visible, even in an unstained preparation, had obvious morphologic defects, which surprised me, because I had collected semen from that bull every Wednesday for the last 10 weeks, and that bull had "passed" his SFT testing each time. Trying to make light of the situation, I looked up at the monitor and said: "Gee, that guy looks like he oughta be at McDonald's". I saw Dr. Sikes flinch slightly at the microscope. Then he arose slowly from his chair, put his arm around my shoulders, and said softly, "<u>that</u> semen came from <u>that</u> patient right there," whereupon he pushed ever so gently on an exit door, revealing a middle-aged gentleman. I hadn't noticed him! I wanted to apologize and beg his forgiveness, but Dr. Sikes wisely advised me to "just go." To this day, I can only hope that the patient didn't know what I meant!

And lastly, here's another cautionary tale, this one about being careful what one wishes for. It involved a dairy of about 400 cows (this was awhile ago), managed by an elderly father and his two 30-something sons. Our ambulatory/herd health practice had for years provided the routine herd checks for general health and reproduction, and we all agreed that something had to be done about the overall infertility in this herd. However, the record-keeping was abysmal, and we had no measuring stick by which to monitor changes. But we weren't worried - we had entered the computer age! Armed with a program that my graduate students had written, we waded into the morass of records, including loose scraps of paper, old DHIA sheets, and breeding cards. After 4-6 months, we began to get a clear idea of the 'progress' they had been making, and for the first time, we were able to show them an objective, computer-generated numbers what and where the problems were most likely coming from.

We were very proud of ourselves, thinking that with our technology, we were one step from going to the moon. Remember, this was the late 1970s/early 1980s.

The father's take on it, however, was different: "What do you need a computer for? I can tell you the problem right now, and the problem is these two boys!"

Those two boys, their father and I were all sitting in the front seat of a Chevy Suburban, waiting for the rain to stop. A family feud erupted, and ended with the two boys walking off the dairy, and their father sobbing in the cab while I wondered, what class did I miss where they taught us how to deal with *this* problem?

It was the first recorded case of computer sabotage in theriogenology.

And now, if there are any among the audience who are trained in the priesthood, please see me afterward where we can discuss hearing my formal confession(s).

Thank you again for the honor of the David E. Bartlett award.

Others to whom I owe a lifetime's debt of gratitude:

Misty Edmondson	Anita Edmondson	Barry Ball
Stuart Meyers	Joan Rowe	Mark, Carolyn Guidry
Doug Byars	Charles Hjerpe	All my grad. students
Nancy East	Rob Pashen	

David E. Bartlett

1917-2014

Paul Bartlett

College of Veterinary Medicine, Michigan State University, East Lansing, MI

David E. Bartlett passed away at home on May 8, 2014. He was born on March 18, 1917 in Bloomfield, New Jersey, to Grace Zeliff and Clarence Durand Bartlett.

David E. Bartlett obtained his D.V.M. from Colorado State University in 1940 and his Ph.D. from the University of Minnesota in 1952. After a year with USDA's Tuberculosis Eradication Division, Dr. Bartlett worked for USDA's National Agricultural Research Center for seven years conducting research on bovine venereal trichomoniasis. He then moved to the University of Minnesota to pursue his Ph.D. From 1953 to 1979, he worked for American Breeders Service (ABS) and continued consulting work with ABS though 1991. He served as "Vice-President of Production and Veterinarian" from 1968-1975 and "Director of Production Division" from 1976-1979. He personally participated in artificial insemination (AI) training of over 3,000 students, several hundred of whom were from foreign countries.

Dr. Bartlett was active in international projects to share artificial insemination technology with many other nations, including Brazil (1958), Mexico (1959), Dominican Republic (1963), Trinidad-Tobago (1969), and the Azores (1979). Dr. Bartlett was a senior veterinarian (Commander) in the US Public Health Service inactive reserves. He was the Chairman of the Organizing Committee for the American College of Theriogenologists and became a Charter Member in 1970 and its first President from 1971-1973. He published over 75 journal articles and chapters and was the recipient of many awards, including the Borden Award (1970), Honored Alumnus from Colorado State (1976), Meritorious Service Award from the Wisconsin Veterinary Medical Association (1977) and Distinguished Service Award from the National Association of Animal Breeders (1980). The American College of Theriogenologists and the Society of Theriogenology have established the annual David E. Bartlett Lecture/Award.

David is survived by his wife Marjorie Cooper Heiner Bartlett, Ph.B., Ph.M. of Madison, Wisconsin and his two sons Dr. David H. Bartlett (Dr. Cheryl) and Dr. Paul C. Bartlett (Pamela), four grandchildren: Dr. Allison Bartlett (Shawn Hannan), Dr. Nathaniel Bartlett (Dr. Lisa Woodson), Andrew Bartlett and Alexander Bartlett and four great-grandchildren: Cooper, Owen, and William Hannan and Lev Bartlett. David leaves behind many friends in Madison at Oakwood Village University Woods, Madison, Wisconsin and in Hawaii where he and his wife Marge visited to avoid at least part of 24 Wisconsin winters. Dave and his wife Marge were members at Luther Memorial Church in Madison, Wisconsin and the Lutheran Church of the Holy Trinity in Kona, Hawaii where they had participated in the building of the church. Dave was active at his residence at Oakwood Village Community Woods where he served on numerous committees and participated in many of the trips and activities. He especially enjoyed his membership in Madison's downtown Rotary Club with its many excellent programs. David was an avid traveler, having visited all 50 states and all continents except for Antarctica.

A memorial service is planned for July 12, 2014 at 11:00 AM at Oakwood Village, University Woods, Resurrection Chapel, 6205 Mineral Point Road, Madison, WI 53705. In lieu of flowers, donations may be made to Luther Memorial Church Foundation, 1021 University Ave, Madison, WI 53715; Oakwood Foundation, Inc., 6201 Mineral Point Road, Madison, WI 53705 https://oakwoodfoundationinc.org/ ; or Lutheran Church of the Holy Trinity, 77-165 Lako St., Kailua-Kona, HI 96740-2266.

The Society for Theriogenology celebrates 60 years

R. S. Youngquist
College of Veterinary Medicine, University of Missouri, Columbia, MO

"We are like dwarfs on the shoulders of giants, so that we can see more than they, and things at a greater distance, not by virtue of any sharpness of sight, or any physical distinction, but because we are carried high and raised up by their giant size."

Bernard of Chartres
12th Century

Summary

The Society for Theriogenology traces its roots to 1954 and celebrates its sixtieth anniversary at this year's annual meeting. The society was founded to meet a specific need of the livestock industry in the Rocky Mountains. Over the subsequent six decades it has undergone several changes in name and grown into an international organization that serves the educational needs of veterinarians involved in the reproductive management of all species served by the profession.

Keywords: History, breeding soundness, professional organization

Formation

The early history of the organization now known as the Society for Theriogenology was described Dr. B.W. Pickett[1] and by Dr. H. J. Hill in a paper he was scheduled to present at the Society's Annual Meeting in 1979 planned for Mobile, Alabama.[2]

Dr. Hill writes of a blizzard that struck north central Colorado in December 1949. By March of 1950, ranchers noticed lesions involving the perineum and scrotum of herd sires.[1] The expertise of the staff of the Colorado A&M Artificial Breeding Service was enlisted to assess the extent of damage to the bull herd in the blizzard area. Dr. Hill and four or five senior veterinary students were dispatched armed with a rudimentary mobile laboratory and artificial vaginas made of "thirty feet of radiator hose hastily conscripted from the supply room of the local Ford dealer".

Unexpectedly, these pioneers found that the severity of lesions was not correlated with semen quality but that ten to 12 percent of the bulls examined did not produce semen of sufficient quality or had physical defects that made them unsatisfactory breeders. Thus, the need for a standardized procedure to assess the breeding potential of bulls was recognized.

Veterinary practitioners and ranchers recognized the need to remove unsatisfactory bulls from the breeding herd and a rancher and Colorado state legislator became a supporter. The state legislature passed a bill giving financial support and the Colorado Bull Testing Service was initiated in the fall of 1953. Bull owners were asked to pay $6.00 for each bull examined.

Evolution

Rocky Mountain Society for the Study of Breeding Soundness of Bulls

In February 1954 about 22 veterinary practitioners joined with the clinicians at Colorado A&M (now Colorado State University) to organize the Rocky Mountain Society for the Study of Breeding Soundness of Bulls. Dr. Eli VonTour of Alliance, NE was elected president and Dr. H. J. Hill was elected secretary/treasurer and appointed editor of the society's journal. The stated purpose of the society was to "disseminate information relative to reproductive physiology, criteria of semen evaluation and physical examination of bulls as applied by the Colorado Bull Testing Service".

Society for the Study of Breeding Soundness of Bulls

The name of the organization was amended to the Society for the Study of Breeding Soundness of Bulls in 1956 to reflect the fact that members were from outside the Rocky Mountain area and the society was given a charter under the laws of Colorado in 1961.[3]

The American Veterinary Society for the Study of Breeding Soundness

Another name change was made in 1963 to the American Veterinary Society for the Study of Breeding Soundness to reflect its stature as a national organization with its focus expanded to include the study of bovine females.[4]

Society for Theriogenology

The name was again changed in 1974 to the Society for Theriogenology in recognition of the society's ever expanding interest in the reproductive processes of all species served by the profession of veterinary medicine.[3]

American College of Theriogenologists

In the 1960's efforts began to establish an American Veterinary Medical Association (AVMA)-recognized specialty in veterinary reproduction. After several unsuccessful attempts, probationary approval was given to the fledgling American College of Theriogenologists (ACT) by the AVMA's House of Delegates at its meeting in Detroit in 1971.[5] This organization was intended to complement the Society and Dr. David Bartlett wrote "I would like to call special attention to the very specific constitutional commitment toward the practitioner of veterinary medicine which are intended to fulfill the intent and desire of recognition and certification of competence of veterinary practitioners in theriogenology. It is the intention that this college will be very closely related to the society and it is the intention that the members of the college will maintain dual membership."

Theriogenology Foundation

The Theriogenology Foundation was established in 2009 for the purpose of providing funds to support and advance theriogenology.

Management

During its first years, the affairs of the society were managed by volunteers who were elected to the various offices and the board of directors. The princely sum of $100 per month was allocated to the secretary/treasurer to fund the office and publication expenses of the society in 1964, provided that much was available in the coffers.[6] By 1971 it became obvious that professional management was required and the Society has subsequently been served by several management organizations.

Garey Management Organization (GMO); 1971-1999

Bob Garey (1971-1986) of Hastings, NE was the first executive director of the society and is credited with establishing sound financial management procedures and providing the organizational skills needed to guide an increase in membership. Upon Mr. Garey's retirement, GMO was led by Don Ellerbee who served the society from 1986 until 1999. No mention of GMO would be complete without recognition of the efforts of Ms. Jan Weiler, a longtime employee of GMO. Jan was the telephone voice of the Society and managed the annual meetings (and most other things). Her dedicated service to the society is well-remembered by many society members.

Walker Management Group; 1999-2002

The business of the society was supervised by Walker Management Group, Nashville, TN from 1999 to 2002.

Franz Management; 2003-present

The current executive director of the society is Dr. Charles Franz of Franz Management, Montgomery, AL.

Continuing education

The purpose of the Society was and continues to be provision of the most current information available. Initially, the focus was on information relative to the evaluation of breeding soundness of bulls but the emphasis has broadened greatly over the past 60 years. The society has met its goal of providing continuing education in a number of ways.

Short courses

When the society was founded, prospective members were required to attend short courses offered at numerous sites throughout the United States for training in the theory and practice of evaluation of breeding soundness. This requirement was dropped after several years when the material was included in the curricula of the veterinary colleges.

Annual conferences

The society, in collaboration with the ACT, sponsors an annual conference which provides members with the opportunity for continuing education. The conference has grown from a single day held in conjunction with the annual meeting of the AVMA to a multi-day event composed of several simultaneous topic tracks. In addition, pre- and post-conference symposia offering indepth training are provided to members and livestock and companion animal breeders.

Memorable annual conferences. All annual conferences are memorable for their educational content and collegial interaction but a few have become etched in the memories of those who attended or tried to attend.

Mobile, Alabama—1979

The annual conference of the society was scheduled for Mobile, Alabama in September of 1979. Unfortunately, that meeting was cancelled by the arrival of Hurricane Frederic in Mobile Bay in time to register for the meeting on the evening of September 12, 1979.[7] Frederic was a category 3 hurricane and raked the Mobile Bay area with winds gusting from 100 to 145 miles per hour from the evening of September 12 until the early morning of September 13. Because of the extensive damage, the name Frederic was retired and will never again be issued for an Atlantic hurricane.[8]

Vancouver, British Columbia—2001

The annual conference of the society was scheduled for Vancouver, British Columbia to begin on the evening of September 12, 2001 in conjunction with the annual meeting of the American Association of Bovine Practitioners (AABP). The attack on the World Trade Center on September 11, 2001 resulted in closure of the airspace over the United States and Canada and airplanes were grounded. Those who were in Vancouver were unable to leave and those who were planning to attend were unable to travel. The president of the AABP wrote that the attack was the defining event of his presidency.[9]

Publications

Journal of the Rocky Mountain Society for the Study of Breeding Soundness. Throughout its history, the Society has used a number of publications to achieve its purpose "to disseminate what information is available currently, relative to evaluation of bulls for breeding soundness, with the sincere hope that the criteria, techniques, philosophies, and facts presented will become the common guide for all who offer such a service to the livestock industry".[10]

The early publications of the society consisted of a series of pamphlets setting forth the philosophy of evaluation of bulls for breeding soundness; Volume 4, number 1 giving the purpose and bylaws of the society and a brief treatise on technique of evaluation; Volume 5, number 1 a detailed discussion of techniques of all methods of semen collection and a paper on infertility in the bull; Volume 5 number 2 the printed papers presented at the society meeting in Steamboat Springs, CO in the fall of 1955.

The fifth publication in the series is Volume 6, number 2 which is the workbook detailing the original criteria recommended by the society for the evaluation of bulls for breeding soundness. These standards were used by members of the society from their adoption in 1956 until the criteria were revised in 1976.[11]

These journals were published at irregular intervals which was explained by the subheading "published when the spirit moves and time permits". Journals of the society were produced in a shed at the Colorado State University Bull Farm by typewriter and mimeograph, stapled and mailed to members. Unfortunately, a flood in the early 1960's destroyed the shed and all copies of the publications.[12]

Proceedings of the Annual Conference. The society has regularly published the proceedings of its annual conferences and distributed them to members as a means to fulfill its mission of providing continuing education for members. More recently, pre- and post-conference symposia have been added to the society's educational offerings and the manuscripts from these presentations are likewise published.

Compilations. Several compilations of manuscripts were collected and disseminated to members of the society as a method of providing continuing education.

American Veterinary Society for the Study of Breeding Soundness: A compilation of current information on breeding soundness evaluation and related subjects. Hastings (NE): American Society for the Study of Breeding Soundness; 1973.

The Society for Theriogenology: A compilation of current information on breeding soundness evaluation and related subjects; revised edition. Soc Therio J Vol VII, 2nd ed. 1976.

Manuals. Species manuals were prepared by experts in their field and distributed to members of the society. A list of the species manuals is presented in the table.

Manual title	Year of publication
Brood Mare	1978
Stallion	1980
Sheep and Goat	1980
Breeding Soundness Evaluation of Bulls	1982
Breeding Soundness Evaluation of the Boar	1983
Cow Manual	1987
Manual for Embryo Transfer	1987
Reproductive Herd Health (Dairy Section)	1988
Sow Manual	1989

Table. List of species manuals produced and distributed by the Society for Theriogenology.

Fact Sheets. In the late 1980's and 1990's, a series of Fact Sheets prepared and reviewed by members of the society replaced the species manuals.

Clinical Theriogenology. In 2009, the Executive Boards of the Society for Theriogenology and the ACT established the peer reviewed quarterly journal titled Clinical Theriogenology as a means to publish the proceedings of the annual conference and to provide an outlet for members to distribute their research results and clinical observations. Clinical Theriogenology is the official journal of the Society

for Theriogenology and the ACT and is available in print and online as a benefit of membership in the society.

Zemjanis Outreach Fund

The Zemjanis Outreach Fund was established to provide partial support for veterinarians from developing countries to travel to the United States for educational purposes.

Awards

A number of awards have been established by the society and ACT to recognize the professional accomplishments of members.

The David E. Bartlett Award and Lecture was established in 1984.

The Theriogenologist of the Year Award was established in 1999.

The Steiner Award for Practitioner Excellence was established in 2009.

Nandi—Symbol of the Society for Theriogenology

In a letter dated August 20, 1982 addressed to Ms. Jan Weiler, Dr. D.F. Walker describes the origin of "Nandi" which has become the symbol of the society.[4]

> "In July of 1956 when Harold Hill was putting the Volume 6, Number 2 publication together, which became the Society workbook, he was inspired by an article in Holiday magazine which carried a picture of "Nandi" and by an inscription to adopt Nandi as the logo. The inscription was as follows: 'From atop Chamundi Hill at Mysore, the giant image of Nandi, sacred bull and symbol of fruitfulness casts his benign gaze upon his worshipful flock.' Aubrey Menen; Holiday, June 1956
>
> I (Walker) was also quite inspired with the picture of the bull and the inscription as it appeared in our journal. While in India I made a trip to Mysore to see Nandi. The statue was quite impressive but I discovered that it was not the same Nandi as appeared in the Holiday magazine, which we had adopted. The Nandi pictured in the magazine and used as the Society logo is located in Oriasa, India and is a copy of the original at Mysore. Apparently the author of the article, Aubrey Menen, selected the wrong Nandi from the picture file when the article was published.
>
> I acquired an exact copy of the original Mysore Nandi and from this a brass casting was made by the brasssmith in our village. I would like to give the brass Nandi to the Society to be a traveling badge of office and pass from chairman to chairman. I will send the Nandi to the Milwaukee meeting. It weighs 22 pounds and will make a beautiful desk ornament for the chairman."

Conclusions

The Society for Theriogenology was born of the need for the veterinary profession to serve the livestock industry for the specific purpose of identifying bulls that were not suitable for breeding. The original focus was narrow and the horizon was close. Over the 60 years of its existence, the society has grown in membership and expanded its focus and horizon to meet other needs of its members and the animal owners they serve. Indeed, those of us who have been privileged to have been members of the Society for Theriogenology have been the beneficiaries of the tireless effort and vision of the founders, those giants on whose shoulders we have been carried high.

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Enhancing the learning experience of students in reproductive science with multimedia platforms

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Abstract

Understanding how information is processed is key to effective communication. Furthermore, available digital technology provides an opportunity to enhance the learning process in a shorter period of time. This paper will highlight research that utilizes instructional design principles in a multimedia platform to teach complex concepts in reproductive science.

Keywords: Student learning, multimedia, teaching, reproductive science

Introduction

Most of us who teach in the field of reproductive science, whether it be basic reproductive physiology, theriogenology, obstetrics and gynecology or various social services such as Planned Parenthood, district health centers, etc., have had little, if any formal training in cognitive psychology and/or how to apply documented instructional design principles to concepts that are very important for people to understand. Almost all of us have learned to teach by mimicking our peers or former mentors.

During the past eight years, we have been developing strategies for using multimedia to enhance the learning experience of students and explain concepts in reproductive science. We have learned that a solid understanding of multimedia instructional design principles is invaluable to developing strong, day-in and day-out instructional designs in our classes and presentations. We have produced various multimedia prototypes and tested these in 11 land grant universities using over 1,500 undergraduate reproductive science students. The U.S. Department of Education through the Small Business Innovation Research (SBIR) program has funded much of this effort.

Students are relying on mobile devices such as cell phones, tablets and laptops to access information at an accelerated rate. Student behavior using these devices are ideally suited for multimedia teaching/learning and a promising opportunity exits. They are embracing nontraditional learning opportunities that can occur anytime and anywhere.

The development of multimedia approaches that explain concepts in reproductive science could significantly reduce the time for learning. Further, multimedia approaches using technology that students already embrace (and that is constantly improving) would potentially result in an equal or better learning experience than traditional approaches. The purpose of this paper is to describe and summarize the possible uses of multimedia as a method to enhance understanding of important concepts in reproductive science.

The specific objectives are as follows:

- describe some of the most important principles of multimedia instructional design
- identify how these principles can be put into practice,
- demonstrate some research outcomes that support the power of a multimedia approach, and
- discuss the outcomes of Beta tests to evaluate presentation of online content units in reproductive science.

Overview of multimedia principles

Understanding concepts in reproductive science require the integration of anatomical, hormonal and behavioral changes over time. Understanding these concepts is obligatory for proper implementation

of reproductive management and reproductive health interventions. A problem that impedes understanding is that as complexity of a concept increases, cognitive load increases. In other words, the amount of cognitive resources needed to process information increases as complexity increases.[1] To minimize cognitive load so that maximum learning potential occurs, instructional design principles must be utilized, especially in a multimedia presentation.[2] Multimedia is defined as the combination of words (written or spoken) and pictures, and can be applied to something as simple as a book or elaborate as an online course.[2] Five foundational multimedia principles for instructional design and how they can be utilized are presented below:

- **The modality principle** incorporates both the visual and auditory sensory channels when presenting information to expand working memory capacity and increase learning.[3-6] We incorporated graphics (visual) and narration (auditory) to maximize information processing in our multimedia presentations.
- **The spatial and temporal contiguity principles** provides explanations or easily-identifiable key words at the same time as information is presented.[5,6] Our multimedia presentations synchronize the narration with the animation. Therefore, explanations describe events as they are happening. Also, labels linked to anatomy or processes are provided, rather than simply showing an anatomical section with numbers or letters that requires students to actively search for the key to understand. In other words, students do not have to search for names and functions to understand the anatomy. Much of the printed literature in reproductive science makes it very inconvenient for students and instructors alike.
- **The signaling principle** directs the learner's focus by emphasizing or highlighting specific points of a concept.[7,8] For example, inserting an arrow on a graphic guides the learner's attention to the specific segment so that the narration of that particular concept can be understood without the learner guessing what he/she should be paying attention to.
- **The personalization principle** indicates that explanations in a conversational format are more effective than explanations presented in a formal format.[9,10] Multimedia presentations with the narration including "I" and "you" personalizes the communication and builds a social connection between the instructor and the learner. For example, a new topic can be introduced by saying "next, you will learn the stages of the estrous cycle" rather than "the stages of the estrous cycle will be described next".
- **The Coherence Principle** states that concise explanations without extraneous information are most effective for minimizing cognitive load.[11,12] Extraneous information can include ancillary videos, background music, sound effects and supplementary written material that do not pertain to the core information. Extraneous information almost always distracts the learner and decreases learning because the learner is trying to sort the information into relevant and not relevant categories rather than focusing directly on the topic. In reproductive science, there are many differences among species. The instructor might try to embed the differences within the core concepts. This significantly increases the cognitive load and often confuses students.

We have applied these principles in the development of multimedia presentations that describe complex concepts in reproductive physiology. Our research and development pathway is presented in Figure 1. The pathway is divided into two sections: 1) single concept (~10-15 min) development and experimentation; and, 2) multiple concept (~60 min) development. The single concept experiments built upon short-term presentations with an immediate measure of knowledge gains. Details of these results are published elsewhere.[13-15] The multiple concept development phase focuses on longer multimedia programs incorporated into an online setting.

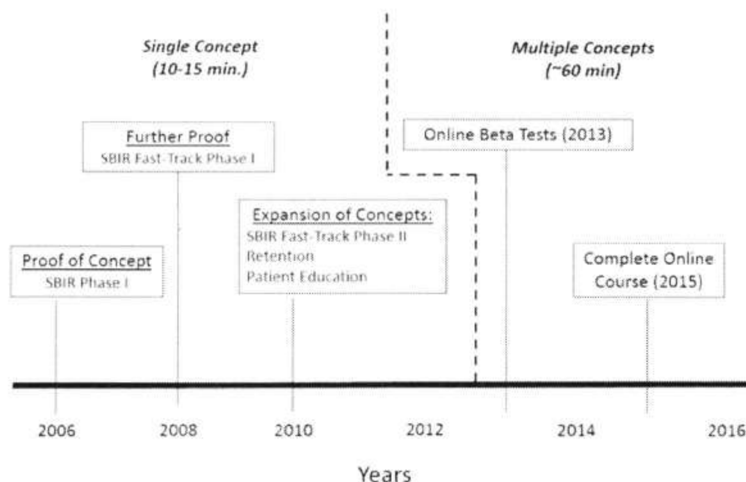

Figure 1. Pathway of multimedia experiments using multiple reproductive physiology topics. The pathway is divided into two phases (single concept and multiple concepts).

Review of single concept research/development

During the single concept phase, we have shown that learning is increased when instructional design principles were employed to describe several concepts in reproductive science. These topics were: *"Mammalian Follicular Dynamics"*, *"The Physiology of Parturition"*, *"The Menstrual Cycle and Oral Contraception"*, *"Assisted Reproductive Technology"* and *"Lactation and Nursing"* (access to multimedia programs are available upon request). All multimedia presentations include the following features:

- 2-D and 3-D animations (animation principle[16,17])
- Synchronized narration (personalization[9,10] and temporal contiguity principles[5,6])
- On-screen labels (spatial contiguity principle[5]) and,
- No extraneous information (coherence principle[11,12])

Overall, the single concept research phase included over 1,400 students from six land grant universities and 122 patients at an OB-GYN clinic. A summary of experimental outcomes is described below.

Multimedia components

Our first research effort compared test scores after students viewed either a multimedia presentation of mammalian follicular dynamics or a traditional lecture captured on video.[13] Students who viewed the multimedia presentation scored 15.5% higher ($p < 0.05$) on tests of the material than students who viewed the videoed lecture (78% and 62.5%, respectively). Furthermore, the multimedia presentation required one-half the time of that for the video lecture. The second experiment (using *"Mammalian Follicular Dynamics"* as the topic) isolated the effect of 3-D animation on student learning.[13] Again, the results of test scores favored ($p < 0.10$) the 3-D component of the multimedia presentation.

Two experiments were then conducted to examine the impact of animation type and narration length on student learning.[14] Students in the animation study were randomly assigned to one of three groups and viewed a presentation on *"The Menstrual Cycle and Oral Contraception"* with no animation, 2-D only animation, or 3-D animation. Type of animation did not influence test scores, indicating that some concepts (like learning the basics of the menstrual cycle and oral contraception) do not require 3-D animation to successfully transfer information. However, some concepts like follicular dynamics

appeared to be dependent on 3-D animation for the learner to successfully process and assimilate the information.

Next, students were randomly assigned to view a presentation describing *"The Physiology of Parturition"* to compare narration length (short narration-14 min vs. long narration-24 min).[14] There was no difference between student test scores for narration length; indicating that students can learn effectively when explanations are concise, even in a short time period. Explanations that are longer than necessary are inefficient for both the instructor and the student.

Knowledge retention

The next research question was, "does a multimedia presentation extend knowledge retention when compared to a traditional lecture"? An experiment was conducted with university students (n = 46) that measured test scores immediately after viewing, one week later, and one month after viewing the presentation.[14,15] Students did not know when the second and third tests would be given so that they did not have an opportunity to study prior to the tests. Again, students in the multimedia group significantly outperformed ($p < 0.05$) students viewing the traditional lecture captured on video. Furthermore, there was a significant ($p < 0.05$) effect for time after viewing. Mean test scores (out of a possible 25 questions) for the multimedia group were 64.32%, 56.52% and 64.52% correct; whereas mean test scores for the traditional video lecture group were 56.88%, 49.56% and 53.40%, respectively, for students tested immediately after viewing, one week later, and one month after viewing each type of presentation. As expected, test scores declined over time, indicating that consistent studying is necessary to maintain knowledge. Interestingly, when the resident professor gave a review session between the second and third test, students in the multimedia group had test scores similar to that of just having watched the presentation. In contrast, the test scores in the traditional lecture video did not return to the original test score, indicating that a brief review of the material following a multimedia presentation is an efficient method for teaching and learning.

Educational background of audience

Another study was conducted to determine if the instructional design used for university students would be effective to people who are not students of reproductive physiology.[14,15] Pregnant women (n = 122, with various ages, parity and educational levels) at Northwest OB-GYN clinic in Spokane, WA were asked to view the presentation, *"The Physiology of Parturition"* or read a booklet containing the same graphics and descriptions and complete a short multiple-choice quiz. Women in the multimedia group significantly outperformed ($p < 0.05$) women in the booklet group. Although not a direct comparison, 15 test items on this test were identical to a previous test given to university students.[14,15] The university multimedia group's score was 88%, while the patient group's score was 83%, indicating that proper instructional design of complex information can be used to teach a wide variety of people, especially when the concepts are applicable on a personal level.

Multiple concept development

During the fall of 2013, we conducted a Beta test with three content units that consisted of multiple concepts at three universities (North Carolina State University, South Dakota University and Montana State University; see Tables 1, 2 and 3). The objectives of this qualitative study were to: 1) test the online platform delivery to determine functionality limitations; 2) determine student reactions and feedback to platform use and instructional design; and, 3) measure student learning and navigational behavior within each content unit. The online platform was developed by SchooX (www.schoox.com; access to the content units available upon request). The platform allowed students to access information at their own pace and monitor their progress in each content unit (i.e., how many segments completed and quiz score/attempts). All professors required the textbook, *Pathways to pregnancy and parturition-3rd ed* in their classrooms.[18]

The content units were: *"Male Reproductive Anatomy and Function"* (NCSU, n = 47), *"Reproductive Cyclicity: Terminology and Basic Concepts"* (SDSU, n = 76), and *"Reproductive*

Cyclicty: The Follicular Phase" (MSU, n = 65). Each content unit consisted of multimedia presentations containing Powerpoint® presentations and illustrations using Bamboo® software. Each multimedia section was synchronized with narration describing each concept. After viewing the multimedia sections in the content unit, students completed a quiz and brief survey. Students were allowed to take the quiz as many times as they wished. Participating instructors agreed to not lecture on the content unit prior to the Beta test. Quiz scores counted towards their overall course grades. Students from NCSU accessed the online content during the second week of the semester, SDSU students participated during the seventh week of the semester and MSU students accessed the online content during the eighth week of the semester. A summary of outcomes is provided below.

- Quiz performance was high (see Table 4):
    - 57.3% of the students scored a 90% or above
    - 16.8% scored 80-89%
    - 5.6% scored 70-79%, and
    - 20.6% scored 60% or below
- Features of the online content unit were ranked according to perceived importance by the students:
    - Over 50% of the students ranked ease of access, self-paced learning and the fact that the content was linked to the textbook as the most important factors
    - Synchronized narration and practice tests were ranked lower in importance
- Overall experience accessing the content units was positive (see Tables 5 and 6):
    - 84.7% of the students ranked the content units as "good to exceptional" (of which, 56.1% ranked the content as "great or exceptional")
    - 14.6% ranked the content unit as fair
    - 30% ranked their content unit as poor

Results from the Beta test are quite encouraging but certainly require further testing for accuracy and precision. Future efforts will include determining: 1) better testing/grading features; 2) a "Frequently Asked Questions" section; and, 3) improved student behavior monitoring to easily identify problem areas within each content section.

Beta test discussion

Quiz scores

Quiz scores were exceptionally high and probably reflected the fact that students could engage the quiz as many times as they liked until a satisfactory score was achieved. In addition, the motivation for obtaining as many points as possible to help with their overall course grade cannot be separated from other factors. Many students ranked the practice quizzes lower in the content unit because they found the answers to be very unforgiving. For example, if students typed in a correct answer that was not in the test bank or spelled words incorrectly, then these were marked as incorrect answers. More possible correct answer combinations will need to be incorporated into the quiz bank for future use to eliminate this problem. This is one limitation of a software-based testing system compared to an instructor personally grading each answer and interpreting what the student meant.

Student navigation behavior

Another advantage to using an online platform to deliver information is the ability to track each student's navigational behavior, something that one cannot do in the classroom. For example, some students might prefer to take the practice quiz first and then access content unit sections only to fill knowledge gaps, while other students might view an entire section and then answer the quiz questions. Each type of behavior might result in increased learning for individual students.

When using the quiz feature, many students accessed the corresponding content several times as they answered the quiz questions, using the multimedia sections as an "open book resource". Only a few

students accessed the content minimally and focused on practicing with the quiz rather than revisiting the content. Some students did not access the multimedia sections at all and instead, chose to read the book and then answer the questions. Student navigational behavior can be used to modify content delivery and platform compatibility so that different studying styles can be automatically accommodated. However, more research and development is needed to determine which features of this type of platform will function the best in an online setting.

Conclusion and future implications

Updated versions of the platform will be built so that the instructional team can better understand where, and perhaps why, students have difficulty understanding. Specific sections of the multimedia presentations that are reviewed repeatedly and correspond to related quiz questions can be pinpointed to isolate "trouble spots" and improve the delivery so that a more thorough understanding is achieved. Additionally, quiz responses can be collected over time to determine which incorrect answer is marked the most and review the corresponding content unit section to determine why students are confused or missed the point. Then the instructor can effectively modify the explanation or test items to reduce confusion and misunderstanding in the future. Our ultimate research and development goal is to validate the components of online delivery that will enable implementation of a highly accurate and efficient multimedia delivery platform to maximize a student's learning experience and understanding of complex processes in reproductive science.

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Table 1. Participation

	NCSU	SDSU	MSU	Total
University enrollment	54	76	67	197
Online enrollment	49	76	67	192 (97.5%)
Online quiz	47	76	65	188 (95.4%)
Online survey	48	59	45	152 (77.1%)

Table 2. Demographics - Class Rank (% Responses)

	NCSU	SDSU	MSU
Freshman	0.0	1.8	0.0
Sophomore	8.3	0.0	2.2
Junior	58.3	7.1	51.1
Senior	29.7	91.1	46.7

Table 3. Demographics - Animal Experience (% of Responses*)

	NCSU	SDSU	MSU
Grew up on commercial farm, animal business	20.8	58.9	37.8
Worked for commercial farm	29.2	39.3	40.0
4-H/FFA	25.0	53.6	64.4
Limited to pets	50.0	23.2	33.3
Worked for veterinarian	68.8	30.4	48.9
No experience	4.0	0.0	0.0

*responses are greater than 100% because students checked all applicable categories

Table 4. Test Performance (% of Students and Mean # of Quiz Attempts)

	NCSU		SDSU		MSU		Overall Mean
	%	# attempts	%	# attempts	%	# attempts	% Students
A (90%)	39.6	3.5	80.3	3.5	52.3	2.0	57.3
B (80%)	10.4	1.3	17.1	2.8	23.1	1.1	16.8
C (70%)	6.3	2.0	1.3	1.0	9.2	1.0	5.6
D (60%)	8.3	1.0	0.0	0.0	6.2	1.5	4.8
F (<60%)	35.4	1.7	1.3	1.0	9.2	8.0*	15.8

*mean number of attempts skewed as one student took the quiz 12 times

Table 5. Would You Recommend This Content Unit to Other Students?

	NCSU	SDSU	MSU	Overall Mean
Yes	40	96.4	82.2	72.9
No	60	3.6	17.8	27.1

Table 6. Overall Experience Ranking

	NCSU	SDSU	MSU
Good-Exceptional	54.2	100.0	100.0
Fair	14.6	0.0	0.0
Poor	30.0	0.0	0.0

% Rank of Exceptional or Great

NCSU	SDSU	MSU
31.3	68.4	68.5

Recent advances in swine reproduction

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Abstract

Accurate estrus detection is an essential component of a successful breeding program in modern swine operations. It is labor intensive, time consuming, and an economically important aspect of the production system. Over the last 20 to 30 years, swine production systems have changed: there are fewer farms, with these farms holding larger numbers of breeding females. Along with this restructuring in the swine industry, application of techniques to improve reproductive efficiency and controlled breeding has occurred.[1] Some of the assisted reproductive techniques now commonly utilized in modern swine operations include artificial insemination, hormonal induction of estrus in gilts, estrus synchronization of sows and gilts, and B-mode ultrasonography.[2-5] Even with the addition of all of these technologies, prediction of ovulation is limited to a range of hours and is still considered a challenge for the swine industry.

In recent years, there have been several advances that have been made to improve the reproductive efficiency of the pig and increase the accuracy of inseminating at the proper time. Some of these advanced techniques include: induction of ovulation, fixed-time artificial insemination, deep uterine and post-cervical insemination, and cryopreservation of semen. These advanced techniques will be presented in this article as well as some discussion regarding production of porcine embryos and embryo transfer.

Keywords: pig, reproduction, assisted reproductive techniques, ovulation, artificial insemination

Introduction

Sows and gilts are currently artificially inseminated (AI) with multiple services (doses) of extended semen based on heat detection after estrus synchronization. Even with good estrus detection methods, it can be difficult to attain maximum fertility results since there is a wide range between timing of ovulation in relation to onset of estrus. According to Soede and Kemp[6] (1997), pigs ovulate 10 to 58 hours after the onset of estrus. Best fertility is attained mating within 24 h prior to ovulation.[7] The ideal time for insemination is 8 to 12 h prior to ovulation.[8] The availability of these time periods provide a breeding window, however, the large variety in hours makes it even more critical for accurate estrus detection with the goal of mating as close to ovulation as possible. The broad challenge is being able to predict when the pig will ovulate.

The basis of most of the recent advances in swine reproduction has concentrated on manipulation of the estrous cycle in order to place semen as close to ovulation as possible. With this in mind, breeding programs are trying to become more efficient without adversely affecting pregnancy and farrowing rates. To increase productivity, research efforts have concentrated on induction of ovulation so that breeding protocols inseminate only once (fixed-time artificial insemination; FTAI) with a normal dose, reduced dose or cryopreserved boar semen.

Induction of estrus

The basis of all assisted reproductive strategies is for the female to be in estrus (cycling) and subsequently ovulate in a relatively predictable manner. With respect to estrus in the pig, there can be variation that occurs with the onset of estrus to ovulation and the length of estrus.[9-10]

Breeding management has concentrated efforts on synchronization of estrus initially to then assist in predicting when ovulation will occur.

Weaning

Traditional management and attempts to synchronize a group of females into a breeding schedule have involved weaning of piglets from lactating sows on the same day. Suckling piglets provide the stimulus for hormonal suppression and therefore, anestrus during lactation. This hormonal suppression provides the sow time for uterine involution, during which, the uterus must undergo a rapid loss of length and weight. This generally occurs during the first two to three weeks of lactation.[11] Fernández et al. (2005) reported that the wean-to-estrus intervals are shortest for sows weaned between three and four weeks after farrowing.[12] Sows weaned at less than 10 days of lactation demonstrate a much longer wean-to-estrus interval and in contrast, a greater percentage of sows that lactate for more than 20 days return to estrus by Day 7. The time of exhibiting estrus following weaning is linked to adequate time for uterine involution.

In general, females will show estrus 3-5 days post-weaning. The benchmark for wean-to-service interval (WSI) is less than 7 days and is directly related to the timing of behavioral estrus in the weaned female. The important thing to realize with this management practice is that sows can have a large variability when they come into estrus and the duration of estrus.[9-10] Kirkwood (1999) has reported that sows that show signs of estrus within a short time post-weaning stay in estrus for a longer duration than those that come in later (> 5 days post-weaning).[13] This means that sows that come in early will ovulate later in estrus than those that come in later as they still ovulate approximately 2/3 of the duration of estrus. Without administration of an ovulation inducing agent, the manager spends a lot of time performing estrus detection and insemination based on standing estrus behavior.

Altrenogest

Altrenogest is an orally active progestogen which inhibits gonadotropin release, imitating the biological activity of progesterone (Matrix®; Merck Animal Health distributed by Intervet American Inc., Millsboro, DE). It does not prevent luteolysis, but it blocks the onset of estrus even after luteolysis and allows for continued follicular growth and development after its removal.[14] In order to synchronize estrus in gilts, Altrenogest is administered orally as a top-dressing in feed at a dose of 15-20 mg/day/gilt for 14-18 consecutive days. Estrus can be expected 5 to 7 days after the last day of Altrenogest treatment.[14-15] The majority of these females have been observed in estrus previously and considered to be cycling.

Altrenogest can be used to synchronize estrus in weaned sows – with most of the efforts being concentrated on primiparous sows as they have the most difficulty in resuming cyclicity post-weaning. There have been several protocols, involving varying doses and length of treatment, examined for treatment of these females. Investigators have compared length of treatment ranging from 4 up to 15 days with the initial day of treatment beginning the day before weaning.[16-18] From review of these studies, it appears as if a longer treatment (15 days total) yielded the most acceptable farrowing rates compared to treatment lengths of 4 and 8 days.[18] Continued research occurs in this area (is ongoing) to determine follicular size and reproductive hormone profiles at various points during and post-treatment with altrenogest in primparous sows.

P.G. 600®

The combination of gonadotropins, follicle stimulating hormone and luteinizing hormone, are used by the female to stimulate follicular development to an appropriate size and maturity to stimulate rupture of these follicles (ovulation). In swine breeding programs, this combination of these naturally occurring gonadotropins is marketed as a product (P.G. 600®; Merck Animal Health, distributed by Intervet American Inc., Millsboro, DE). This product is composed of 400 IU pregnant mare serum gonadotropin (PMSG) and 200 IU human chorionic gonadotropin (hCG) and is labelled to induce estrus in prepubertal gilts and return anestrus sows to heat. The PMSG acts like FSH and stimulates follicular development and the hCG acts like LH and induces ovulation. But since these compounds are given simultaneously, there is not an accurate time that ovulation is induced.

Administration of P.G. 600® to sows on the day of weaning stimulates ovarian follicle development and results in a greater expression of estrus with a shorter wean-to-estrus interval.[19] Additionally, this leads to an increase in estrogen, an LH surge, and improves return to estrus in multiparous sows and primiparous sows.[19-20] The average time of ovulation is not normally affected by treatment with P.G. 600® and sows will typically ovulate 45 hours after the onset of estrus.[19]

Induction of ovulation

The use of exogenous hormones to induce ovulation is a common practice in other species such as the equine where follicular development is visualized using B mode ultrasonography.[21] Within the past 10-15 years, ultrasonographic imaging of the ovarian structures of gilts and sows has become more common and can be followed throughout the estrous cycle.[22-24] With this increased information regarding follicular dynamics and assessment of formation of corpora lutea, the timing of when ovulation occurs can be more accurately predicted. In order to predict ovulation, breeding managers use products such as GnRH or LH-like compounds in combination with estrus synchronization protocols.

PMSG + hCG

Although these hormones are used in combination in the P.G. 600® product, they are used in a very specific protocol for the induction of ovulation. The ovulatory process involved in an estrus synchronization program using P.G. 600® is not as exact as when these hormones are given separately and at specific times.[11] For induction of ovulation, the PMSG is given to stimulate the follicles to develop (follicular development) and then the hCG is given to cause ovulation. PMSG (usually in the form of P.G. 600®) is given to the sow at weaning or day 15 of the estrous cycle and the hCG is given 80-96 hours later.[25] Various breeding programs have been implemented after ovulation is synchronized and may involve single or multiple inseminations.

GnRH analogues/agonists

Gonadotropin releasing hormone (GnRH) causes the release of FSH and LH from the anterior pituitary and has been used in a variety of species to induce ovulation.[11] The use of GnRH in swine breeding programs has only recently been re-evaluated for its effectiveness at inducing ovulation and application in fixed-time AI programs.[26] The administration of GnRH for induction of ovulation has involved intramuscular injection as well as deposition of a gel intravaginally.[27-30] The injectable formulation (Receptal, Intervet, Angers, France) contains 10

μg buserelin, a GnRH analogue, that has been administered at between 77 and 120 h post-weaning or synchronization protocol to induce ovulation. Sows treated with 10 μg buserelin at 86 ± 23 h after weaning and inseminated 30 to 33 h later had farrowing rates (87% vs. 84.5% in treated vs. control, respectively). Additionally, similar litter sizes (13.6 ± 3.8 vs. 13.7 ± 3.2 in treated and control, respectively) were reported.[28,30]

The transvaginal formulation of GnRH may be the preferred route of administration as it would potentially minimize injection stress, carcass quality effects, and reduce the loss of GnRH from the site of deposition.[29] The GnRH intravaginal gel product (Ovugel®; JBS United Animal Health; Sheridan, IN) contains triptorelin acetate which is a GnRH agonist. It contains 100 μg of triptorelin and is administered intravaginally 96 h post-weaning.[28-30] According to the label, a fixed-time AI is performed approximately 24 hours post-treatment (day 5 post-weaning). Further studies are in progress to determine if including a gonadotropin at weaning or increasing the dose of triptorelin to 200 μg could improve the synchrony of follicular development and subsequent induction of ovulation so that FTAI breeding programs can be implemented.[29]

Artificial insemination techniques

Most swine breeding programs adopt the strategy of administering two to three individual inseminations, every 12-24 hours after the detection of estrus. Performing multiple inseminations can be critical due to the relatively short viability of oocytes and spermatozoa in the female reproductive tract and also because time of ovulation in the sow is highly variable, and unpredictable.[31] Fertilization results are highly dependent on the time of insemination relative to ovulation; however, the moment of ovulation may vary between 35 to 45 hours after the onset of estrous behavior.[6] Variation in the onset of estrus to ovulation interval limits the chance that insemination is occurring close to the optimal time, within 12-24 hours of ovulation.[11] Breeding managers have increased the possibility of achieving this goal by performing at least 2 artificial inseminations with 3 billion sperm cells during standing estrus. This practice can be time consuming and many producers are interested in reducing the number of sperm cells in an insemination dose as well as performing a single insemination without having negative effects on reproductive parameters (farrowing rate and litter size).[29]

Single fixed-time insemination

With the advancement in protocols to induce ovulation in sows and gilts, the modern swine producer is interested in performing a fixed-time artificial insemination (FTAI) and still achieve high farrowing rates and litter sizes. Many of these breeding strategies have depended on the success of the protocol used to induce ovulation and the method of insemination – traditional cervical, post-cervical, or deep uterine insemination.

Cervical insemination

Traditional AI in swine has involved the placement of the insemination catheter into the cervix and "locking" it into the folds – similar to that of the glans penis of the boar.[31] Semen is deposited via gravity flow combined with contractions of the female reproductive tract, under the influence of estrogen and oxytocin, into the cervix. The semen then migrates up the uterine horns into the oviducts where fertilization occurs. Because of backflow in the post-insemination period, catheters have been designed to allow for post –cervical and deep uterine semen deposition.[32-33]

Post-cervical insemination

This method of insemination has the goal of depositing semen in the uterine body. The main obstacle that must be overcome with using post-cervical insemination (post-CAI) compared to the traditional cervical approach is the cervical folds. This methodology introduces an inner catheter into the uterus by placing it in the lumen of the standard catheter that locks into the cervix. With the success of the design of these catheters, further investigation occurred with decreasing the dose of sperm cells (1-1.5 x 10^9 sperm in a total volume of 30-50 ml) without a significant effect on farrowing rates or litter sizes.[34-37] Studies with this method allowed for further development involving boar semen, fixed-time AI, and deposition of semen further into the uterine horn.

Deep intrauterine insemination

Deep intrauterine insemination (DUI) is the deposition of semen further up the uterine horns compared to post-CAI. There have been various devices designed to accomplish this goal, with the current one being similar to that of the post-CAI catheter, but has a longer length (1.8 m vs. the 15-20 cm in the DUI and post-CAI, respectively). This technology has allowed more opportunities to use a 20-fold reduction in the number of sperm as well as volume and still achieve acceptable farrowing rates and litter sizes. It has been shown that sperm deposited deep into one uterine horn will migrate to the contralateral horn and fertilize oocytes that were ovulated from that ovary. [38] This technology definitely lends itself to be a useful strategy for using other technologies such as frozen semen or sex-sorted semen.[34]

Laparoscopic insemination

The most advanced method of insemination currently being developed for use in swine is the use of laparoscopic technique to inject a very low dose of sperm cells directly into the oviduct. This technology has limitations as it is not currently considered a field procedure as the sow needs to be anesthetized and specialty equipment is necessary, but could certainly be utilized by breeding companies to advance genetic progress. This technique allows visualization of the oviducts of the female pig and placement of 0.3 to 0.5 x 10^6 sperm into the oviduct. If higher doses of spermatozoa are used, polyspermy has been noted and this can be detrimental to the development of the porcine embryo. This technology shows the most promise when using sex-sorted semen.[34]

Advances in semen processing

Boar semen is currently being collected and extended to a dose of 3 x 10^9 in 80-100 ml for artificial insemination via cervical methods. As discussed previously, there has been interest in reducing the number of spermatozoa in each insemination dose.[32] This would increase the efficiency of the boar:sow ratio resulting in the reduction of boar numbers within the stud facility. With the reduced dose and volume, cryopreserved semen could be utilized. Frozen boar semen has the advantage over fresh semen in that it has an indefinite storage life. Other advantages of frozen boar semen includes the usage of semen from animals from all over the world, the banking of superior genetics, and offering another level of biosecurity when introducing new genetic material to a herd.[34,39]

The main issue that needs to be addressed with the use of frozen semen in the swine industry is the cold-induced damage that occurs to boar sperm. The plasma membrane of boar spermatozoa is very sensitive to the extremely cold temperatures required for cryopreservation as

well as ice crystal formation during the thawing process.[34,39] Investigators have concentrated their efforts to improve the actual freezing process, the cryoprotectant in the extender, and the components of the thawing extender.[39-40] Until these aspects are improved, frozen-thawed boar sperm will be a research tool or utilized by genetic companies as the its lowered reproductive efficiency will not allow it to be used commercially although there is some very promising research using frozen-thawed boar semen and FTAI.[41-42]

Advances in other biotechnology

The use of assisted reproductive technologies such as *in vitro* production of embryos, cloning, and embryo transfer has been investigated in the pig for several decades. The success of these biotechnological techniques has been fraught with many difficulties.[32] Pig oocytes and embryos do not develop and mature well in vitro, polyspermic fertilization still occurs at a high rate, and cryopreservation of porcine embryos is subpar. The main reason for much of the failure is the high lipid content within the cytoplasm of these cells. There are a couple of review articles[43-44] discussing the advances in the technologies involved in swine *in vitro* production technologies and cryopreservation of porcine embryos. Continued research in these areas will likely yield improved results in the future.

Conclusion

With artificial insemination being near 100% in the swine industry, advancements to improve the reproductive efficiency of the female as well as the male will continue. There needs to be a high level of success for an assisted reproductive technology to be adapted by breeders/ producers. The industry has become accustomed to high farrowing rates (>85%) with large litter sizes (> 12 piglets). Although producers are interested in implementing new technologies, they are not going to accept a decrease in their reproductive parameters. Some of the technologies discussed in this article have promise in the industry: induction of ovulation and fixed-time artificial insemination may become as common as that of bovine reproductive programs. There is still a lot of room for improvement in the use of frozen semen and embryo programs before they will be used commercially.

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Miniature pet pig reproduction

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Introduction

The introduction of miniature and pot-bellied pigs in the U.S. began in the 1980's and their popularity remained for a number of years and then subsided until about three years ago. Their popularity in Europe was preceded by their re-introduction to the U.S. – and the first place that they gained popularity was Hollywood. Actresses treated these pets the same as they did miniature dogs – carrying them around in small bags and dressing them in fashion ensembles. Due to their unique personalities and similarities to dogs, the popularity of miniature pigs as pets has continued to climb and the need for veterinarians who are willing to provide health care and understand their reproductive anatomy and physiology has increased. The aim of this paper is to provide a brief overview of the reproductive characteristics of the pot-bellied or miniature pig.

Keywords: Pot belly, pot-bellied, pig, reproduction

Puberty

An intact male can become sexually active as early as 6-8 weeks of age. He will express mounting and dominance behavior over his pen mates, other pets, and household objects. Owners may notice the boar exteriorizing his penis, but more commonly they notice "a swelling on the abdomen or belly near the umbilicus" that is present and then goes away. This swelling is likely accumulation of fluid into the boar's preputial diverticulum. The fluid usually has a foul odor and is generally composed of urine and semen. Many resources believe that this fluid is used to lubricate the penis during coitus while others consider this fluid to be an attractant for females. While it may appear that this strong odor may attract some females, the major fluid that contains pheromones in the boar is saliva.[1] When excited, the boar will begin to chomp his jaws and increase production of androgens in the saliva – he will appear to be frothing from the mouth.

Both male and female pigs will need to be of adequate size in order to come into puberty and begin breeding. The general rule is that they need to be two-thirds of their mature body weight for their breed. It has been documented that around 12 to 16 weeks of age, a female pig will go into her first estrus and will continue to cycle every 21 days (range 18-24 days).[2] A gilt may begin showing signs of estrus sooner if she is housed in a small group of other pigs or has contact with a boar. Her vulva may begin to swell and become reddened and she will respond with a lordosis response when pressure is applied to her back as would a domestic gilt. Her behavior will be the most noticeable: the gilt may eat less while in estrus, she will have increased locomotor activity, and seek out affection from other pets. Also, she may become increasingly ornery towards owners (increased vocalization in the form of squealing, rooting and biting behavior towards owner's legs) and many animals until she goes out of estrus, which generally lasts one to three days.

Pre-breeding recommendations

Owners who have obtained miniature pigs with the intent to breed need to consider a variety of reproductive characteristics. The female needs to have at least eight to 12 functional teats in order to raise a litter. Additionally, she needs to be at an appropriate body condition and have shown signs of estrus at least once prior to breeding. Waiting until her second or third estrous cycle will increase the number of ovulations and, therefore, the number of piglets. Another practice that could increase the number of ovulations is that of "flushing". Flushing involves the increase in feeding rate from 2% to 3% of their body weight 10-14 days prior to breeding.[3]

Other considerations involve administration of pre-breeding vaccinations to reduce chances for infertility or abortion. The primary agents included in this vaccine are: porcine parvovirus, *Leptospira* (*canicola, grippotyphosa, hardjo, icerohaemorrhagiae, pomona +/- bratislava*) and *Erysipelothrix*

rhusiopathiae. The vaccine is generally administered three weeks apart with the second dose two to four weeks prior to the animal's initial breeding and then two weeks prior to subsequent breedings to both males and females. Other health recommendations involve a routine deworming program as well as general health examinations.[4-7]

Breeding management

Pigs generally do not have problems showing signs of estrus or receptivity to mating. Some owners may want to have litters at particular times of the year and want to control the time that the females come into estrus and are subsequently inseminated. This is generally not a common practice in breeding miniature pigs as the owners generally let them breed naturally. If a breeder needs assistance and would like to control the estrous cycle through a pharmacological method, then products that have been approved and used in domestic swine may be used. It has been reported[7] that estrus can be induced in prepubertal gilts using a combination of 400 IU pregnant mare serum gonadotropin and 200 IU human chorionic gonadotropin (P.G. 600®, Merck Animal Health, Summit, NJ), but the author does not have experience with this as the females generally come into their first estrus without difficulty. Additionally, if a female comes into estrus and the owner "misses her", they can either wait for her to come back into estrus in 18-24 days or try administration of prostaglandin after day 12 of her cycle. The pig's corpora lutea are not responsive to prostaglandin for luteolysis until after this day of the estrous cycle.[8] The author does not have any experience with the use of altrenogest to manage estrous cycles of miniature swine.

Females that are exhibiting signs of estrus will seek out the male. Upon introduction of the male, she will show interest towards him. There will be a period of courtship that occurs where both parties will grunt and growl at each other and then the female will stand to be mounted by the boar. The boar may be initially cautious and make nose-to-nose contact before moving to the vulvar area. He may root at the abdomen prior to mounting to ensure that the female is properly stimulated and willing to stand. Depending on the experience of the boar, it may take a few attempts to mount the female. He will generally begin thrusting prior to achieving intromission. The act of coitus may take up to 10-15 minutes as the boar locks into the cervix and ejaculates. After a mating has occurred, the male and female may spend time together in the same area. Multiple matings may occur during the time she is in estrus.

Females ovulate approximately two-thirds of the way through estrus (which is about 1-3 days in length). To increase litter size, the female should be bred at least twice daily until she does not stand for the male.[5] Many owners will likely leave the male and female together during the time that the female is exhibiting signs of estrus to ensure that multiple matings occur. If a male is not able to breed the female for various reasons, hand assisted mating may be attempted or the breeder will have to make another plan for breeding.

Artificial insemination can be performed in miniature swine, but the size of the pipettes used for breeding domestic pigs may be too large. Boars can be trained for semen collection from phantoms, using feed sacks, or other objects that mimic a female in standing estrus. Many boars have high libido and an innate mounting instinct so they can be easily trained to mount and semen collected similar to that of a domestic boar.[9] Evaluation of the semen should consist of gross evaluation for color and opacity, microscopic evaluation for motility and sperm cell morphology, and concentration of sperm cells in the ejaculate. The semen could be extended with commercial swine extender if the ejaculate is to be used for breeding of multiple females.

Pregnancy and gestation

The gestation length for a miniature pig is the same as a domestic pig: three months, three weeks, three days or ~114-115 days. Litter sizes will vary from four to 12 piglets with an average litter size of 5.1 piglets. The majority of losses during pregnancy occurs during the first trimester and can be affected by heat stress, nutrition, and other stresses that affect the general health of the pig. In general, piglets will be viable when delivered after 110-112 days of gestation.[5,7]

During gestation, sows need to be fed a diet specific for her energy, protein, and mineral needs.[2] There are commercial diets for miniature swine that can be used to meet these needs – the feeding of a combination of adult and youth diets should meet these needs for the gestating or lactating female. The sow or gilt should gain up to 20% of her body weight during gestation, with the majority of this being during the last trimester due to piglet growth.

Pregnancy diagnosis

Many breeders will assume that their gilt or sow is pregnant when she is not receptive to breeding by the male on her next estrus after mating. They may or may not have a relationship with a veterinarian who could perform pregnancy diagnosis via transabdominal ultrasonography. This can be performed beginning as early as 19-20 days post-mating, but most examinations will occur around 28-30 days after mating.[10] The gilt or sow can remain standing for the examination. A 3.5-5 mHz transducer can be used on the abdomen and placed medial to the flank fold and pointed towards the bladder. A positive pregnancy diagnosis will consist of identifying hyperechoic fetuses within the hypoechoic amniotic vesicles in the uterus (Figure). With an accurate breeding date, the farrowing date can be determined. After 30 days, the growing piglets can be monitored via ultrasonography or radiographs to ensure that they are developing normally.

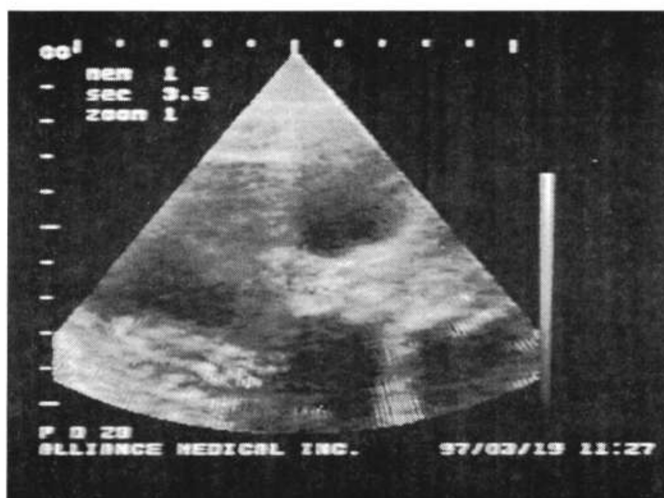

Figure. B mode ultrasonographic image of a pregnant sow ~ 25 days of gestation.

Parturition and neonatal care

It is generally recommended to limit the amount of exposure of the gilt or sow to other pigs or many visitors to the house for two weeks prior to farrowing to limit the exposure to pathogens. It is also a good idea to limit this exposure during the first few weeks after farrowing. Breeders should prepare a farrowing box in preparation for the birth of the piglets. This box should be kept as clean as possible and could contain some small soft bedding. It should be a warm and dry place that is free of drafts and out of the way from the rest of the household and pets. A heat lamp or pad is recommended to keep the piglets warm after they are born as it can be difficult for them to maintain their own body temperature. Be sure to keep electrical cords out of the reach of the sow and piglets as they may accidentally chew on the cords and become electrocuted. Be sure that the sow can find a place to remain cool as her environmental temperature requirements (60-70°F) are much cooler than that of the piglets (92-95°F).[4,11]

Near the time of farrowing, gilts and sows will begin nesting and becoming increasingly restless. This behavior may occur 12-24 hours prior to farrowing. The mammary glands will increase in size a few days prior to farrowing and form a sticky secretion at the end of the teats. The ligaments around the perineal area (tail head) will begin to soften and the vulva will become enlarged and elongate.

During the first stage of labor, the female becomes restless and withdrawn and her breathing may become rapid. She will go to her nest/farrowing box and will not be responsive to food or treats. When she is in the second stage of labor, the rupture of the chorioallantois has occurred and strong abdominal contractions will ensue. Piglets can be delivered in anterior or posterior position and will generally occur every 15-10 minutes. It can take up to four hours for a normal litter to be born – expect that this will be longer for gilts as they will likely rest longer between piglets. The placentas of the piglets can either be passed with each piglet or group of piglets or can all be passed at the end of farrowing.

When monitoring a sow that is farrowing, keep the area clean, warm and as dry as possible. A sow that becomes alarmed or disturbed during farrowing may halt the process and this could lead to a dystocia. Dystocia is not common in pigs (less than 1% of farrowings),[5] but could be more of a concern in the pot belly pig as genetic selection allows smaller females to be bred early and piglets may not pass through a small pelvic diameter. First litter females and older sows are the most likely ones to experience uterine inertia and subsequent dystocia. The uterus may not contract and move piglets through the uterine horns. Females should be examined for any obstruction within the birth canal if a piglet is not delivered after at least three hours of abdominal contractions or it is longer than one hour between piglets. This should always be performed prior to administration of oxytocin. The additional administration of calcium gluconate (SQ) will assist with uterine contractions and delivery of any retained. Causes of dystocia will be discussed in more detail in the "Reproductive disorders" section of this article.

During the postpartum period, the sow should be monitored closely. If this is her first litter, she will likely be nervous and anxious with respect to her piglets and could savage them. These sows may need to be sedated with a phenothiazine tranquilizer such as acepromazine for her to accept the piglets and allow them to nurse. An additional concern regarding the sow is whether she is producing enough milk for the piglets. The sow should lie quietly while the piglets nurse. She will generally grunt to her piglets at the time of feeding to let the piglets know that she is letting down her milk. If she is agalactic, she may move away from the piglets or lie on her mammary glands so that piglets cannot nurse. The mammary glands should be monitored for signs of mastitis and agalactia and treated appropriately with antibiotics, anti-inflammatories, and oxytocin. Additionally, the sow's appetite, general attitude, and rectal temperature should be monitored closely during the first week postpartum. If the piglets need to be fed, they can be fed using a syringe or a pet nursing bottle. They should be fed 10% of their body weight (25-40 ml) at each feeding every four to six hours during the first 12 hours of life. This will ensure adequate immunoglobulin transfer to the piglets. If the sow does not have adequate colostrum, then cow colostrum can be used as a substitute.[2,11]

It is imperative that piglets be fed frequently (every three to four hours) as they do not have adequate fat stores and can die of starvation. Additionally, they need to be kept warm so that they will be able to adequately absorb the nutrients from their gastrointestinal tract. If the piglets are not able to nurse, they can be fed via a stomach tube or supplemented with oral glucose.[2,4,11] Once they are nursing well and gaining weight, they can be transitioned to pan feeding by four to five days of age. Overfeeding can result in the piglets developing diarrhea, which can lead to dehydration. Their feeding should be adjusted as to how they are gaining weight and tolerating feedings if they are not nursing the sow.

Common reproductive disorders

Dystocia

As discussed previously, dystocia is not very common in swine. The clinical signs associated with dystocia and indications for intervention include: gestation over 115 days, prolonged stage one of labor and no progression to stage two, straining and contractions associated with stage two, but no piglets are delivered, delivery of one or two piglets and signs of labor cease, longer than one hour between piglets, and a foul discharge coming from the vulva. There are a variety of causes of dystocia and manual removal of piglets can be difficult in these animals due to size constrictions. If an obstruction is not determined (either by palpation of the vaginal canal or via ultrasonography/radiography), then oxytocin (10-20 IU) can be administered in the muscle to stimulate uterine contraction and expelling of piglets. If

two doses 30-60 minutes apart does not result in delivery of the piglets, then a cesarean section is recommended.[5,7,11]

The procedure for cesarean section in the pig has been described in various sources.[4,12] Once the pig is appropriately anesthetized (+/- epidural anesthesia), a paramedian approach (dorsal to the mammary gland) has been described as to reduce the chance of dehiscence from vigorous nursing from the piglets. Piglets are introduced to the gilt or sow once she has recovered from anesthesia and will lie quietly for the piglets to nurse. Even with a normal birth, piglets are still at a risk of being crushed by being laid on – this may be more of a concern with a female that did not give birth to them via the vaginal route. Gilts or sows should be administered non-steroidal anti-inflammatories and analgesics post-operatively and monitored closely in anticipation that she will be calmer with the piglets if pain is controlled.

Inguinal hernias and cryptorchidism

Both of these conditions occur primarily in male piglets and can be recognized prior to weaning at around six weeks of age. Breeders will generally not attempt to castrate these males. Both of these conditions have been considered to be congenital defects in the domestic pig and should not be used for breeding. Repair of the hernia is recommended at castration. Abdominal exploratory surgery is generally recommended for removal of retained testes. Descriptions of these surgical procedures are available in many of the large animal surgery text books.

Uterine and ovarian masses

It is common for geriatric sows to develop masses on the reproductive tract. The procedure for ovariohysterectomy (OHE) has been the recommended treatment. Various approaches have been described: ventral midline, paralumbar (flank), ventrolateral, and paramedian. For a routine oophorectomy, a paralumbar approach may reduce many of the complications observed post-operatively in pigs. The most common complication has been hemorrhage or development of adhesions which lead to potential gastrointestinal disorders. The author prefers the ventral midline approach as an OHE is the most common surgical procedure that will remove all parts of the reproductive tract that could develop into benign or malignant masses. One should note that the broad ligament of the pig can be quite thick and vascular – this is one of the primary reasons that the author prefers the visualization provided by the ventral midline approach.

Miscellaneous anomalies

There are a variety of other anomalies and disorders that can affect the reproductive tract of miniature swine that also affect domestic pigs. Some of these include: persistent penile frenulum, prolapsed penis and prepuce, preputial diverticulitis, vaginal prolapse, uterine prolapse, and urethral obstruction.[12,13] All of these anomalies are diagnosed and treated according to the severity of the condition and decisions are then made on the potential to affect the individual's breeding capacity.

Conclusion

The author hopes that the information provided will assist reproductive specialists in increasing their knowledge regarding pot-bellied and other miniature pigs. Additionally, this information should provide a little more confidence in providing reproductive services to this species in their practices.

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Appendix

Miniature Vietnamese Pot-bellied Pigs: These miniature pigs represent probably the most popular breed owned today. They have a docile disposition, in general. The author has worked with a number of these pigs that will bite and "attack" the legs of people that they do not know or trust. Their exaggerated pot bellies and swayed backs are completely normal and healthy. Their average height is about 16 - 20 in. and their average weight can be over 100 lbs.

Juliani (Painted Miniature) Pig: These little guys are truly miniature, averaging about 12-16 inches and weighing 15-60 pounds. Like the pot-bellied pig, they have a gentle disposition and are quite playful. The mini Juliana has a longer nose, lighter boned body and longer legs then a pot-bellied pig. They have more of a straight back with less of a belly. The mini Juliana is usually spotted.

African Pygmy or Guinea Hog: These miniature pigs weigh in at an average of 20-40 pounds and reach an average height of 14 to 22 inches. They are active, alert and highly intelligent. In contrast to the pot-bellied pigs, African Pygmies have straight backs.

Kunekune: A small breed of pig from New Zealand. The Kunekune pig is relatively hairy with a pudgy build and may bear wattles hanging from their lower jaw. Color ranges include black and white, ginger, cream, gold-tip, black, brown and tri-colored.

The miniature pig sizes:

"Potbelly Pig": 18 - 26" weigh up to 200 lbs

"Miniature Potbelly Pig": 16 - 20" weigh up to 100 lbs

"Toy Pig": 14 - 16" (Can be a combination of two breeds). These pigs will be many different colors as well to include chocolate, spotted, pink, and red.

"Micro pig": 12 - 16" This is a new breed in itself, you will notice a different body type than the pot-bellied pig and "a longer, squared off nose, straight back, smaller tummy and longer leg."

"Mini Micro Pig or Teacup": 15" and under, same as the micro in looks but smaller. The true ones that stay this small are very rare.

"Mini Juliana Pig": 10 - 16" This is a breed in itself. Unlike the pot-bellied pig, the Juliana is very delicate boned and has a long nose and has spots. Also knows as the painted pig or spotted Juliana. This is the smallest of all of the mini breeds.

Common practices in management of breeding dogs and puppies: a survey of 461 dog breeders

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Introduction

The American College of Theriogenologists (ACT) and Society for Theriogenology (SFT), in 2012, developed a position statement on management of breeding dogs, based on review of the literature available at that time.[1] There were few studies identifying best practices for housing, pre-breeding examination and testing, vaccination and parasite control, and breeding management for bitches and stud dogs. Similarly, there are few studies identifying best practices for raising puppies including best whelping boxes and sources of supplemental heat, diet and age at weaning, vaccination and parasite control, and placement.

The author presented a massive open online course (MOOC) in spring of 2013. The course enrolled 8796 students. Three thousand five hundred four completed a pre-course survey and of those, 1328 (37.9%) identified themselves as dog breeders. The post-course survey was completed by 554 of those who had completed the pre-course survey. The author solicited those who had completed the post-course survey to participate in an anonymous large-scale survey of practices regarding management of breeding dogs and puppies (Figure 1). Breeders were encouraged to pass along information about the survey to their breed clubs and other related organizations.

Results and discussion

Demographics of participants

Four hundred sixty-one people participated in the survey. Three hundred sixty seven did not have a job in health care. Eleven were veterinarians, 29 were veterinary technicians, and 54 were employed in human health care. Forty-seven respondents were from the United States . There were eight respondents from the United Kingdom, six from Canada, two from Australia, two from the Netherlands, and one each from Austria, Brazil, Colombia, Finland, Germany, Greece, Hungary, India, Lithuania, Norway, Poland, Russia, Slovakia, South Africa, Sweden, Thailand, and Ukraine.

Four hundred forty three identified themselves as hobby breeders. Six were commercial breeding operations, four were guide dog facilities, and 29 reported themselves as "other"; this total number is greater than the number of participants who completed the survey because some participants counted themselves in more than one category.

Demographics of dogs in the facilities represented

One hundred nineteen dog breeds were represented (Table 1). Some participants expressed concern about animal rights groups or other parties inappropriately using data provided, and so chose not to provide information about number of dogs in their facility. Number of intact bitches ranged from one to 74, with a mean of 4.1 and a median of 4.0. Age of intact bitches ranged from less than 1 to 14 years of age, with a mean of 3.1 years and a median of 3.0 years. Number of spayed female dogs in the facility ranged from zero to 12, with a mean of 1.7 and a median of 2.0. Age of spayed female dogs ranged from 1 to 17 years, with a mean of 8.0 years and a median of 6.0 years. Number of intact male dogs ranged from 1 to 20, with a mean of 2.6 and a median of 2.0. Age of intact male dogs in the facility ranged from less than 1 to 15 years, with a mean of 4.1 years and a median of 1.0 years. Number of castrated male dogs ranged from zero to 10, with a mean of 1.7 and a median of 1.0. Age of castrated male dogs ranged from 2 to 12 years, with a mean of 7.1 years and a median of 7.0 years. In general, facilities were more likely to house bitches than stud dogs as intact animals but were equally likely to keep spayed females as they were to keep castrated males on the premises.

Husbandry of breeding dogs

Breeding dogs were housed exclusively indoors (n=342), exclusively outdoors (n=29), equally indoors or outdoors (n=120), or variably indoors and outdoors depending on season (n=23). Data were

collected regarding size of enclosures but could not be collated because the question was written too broadly and participants gave widely varying responses without delineating if they were describing cage-like enclosures, runs, or exercise yards, and whether a given space was used to house a single dog or groups of dogs. Many dogs were described as being loose in the house, living as pets, and only being crated at night, when the owner was away, or when the female was in heat. Three hundred twenty-seven respondents described their dogs as being group-housed while 163 housed their dogs singly. It is unfortunate that more information could not be collected about housing as there is a paucity of information in the literature in this regard.[2] General recommendations include housing dogs in groups when possible and separating males from estrous bitches.

When asked if outside dogs were permitted into the facility and if so, how they were managed as brought into the facility, 23 respondents never permitted in outside dogs with many of those respondents citing specific concerns about disease exposure for pregnant bitches or new pups; 15 quarantined newcomers for a varying lengths of time, especially if there were any overt signs of disease; 59 housed newcomers separately but did permit some interaction with their dogs, citing specific variations in how dogs were introduced depending on whether they were there just for breeding or were intended to be long-term residents of the facility; 40 immediately permitted interaction with residents dogs in the facility, with many stating that they would only permit outside dogs into the facility if they knew the owner or source, knew the dog's health history, or had come from their breeding; 27 immediately permitted interaction with no caveats; and seven stated that it depended on the individual dog's temperament, health history, reproductive status (in heat or not), and source. For those that required some sort of health check, six required proof of vaccination, three required negative brucellosis test results with no time frame specified, three required a check-up by a veterinarian, two required a 10-day quarantine at a veterinary clinic, two required evaluation and/or treatment for internal and external parasites, and one required proof of rabies vaccination only. A lack of knowledge of general principles of bioexclusion was demonstrated in these responses, as there is no specific way of knowing that dogs owned by people known to these breeders or dogs originally from their breeding are any less likely to be carrying communicable diseases.

Traveling with or shipping dogs to other countries was not done by 51 of the respondents and was reported by 144 of the respondents. When asked to which countries dogs most commonly traveled, 67 reported travel to Canada; 26 to the United States; 18 to Germany; 14 to Sweden; 12 to Finland and to the United Kingdom; ten to the Netherlands; nine to Denmark and to Mexico; eight to France; six to Australia, Belgium, Brazil, and Italy; five to Norway and to South Africa; four to Austria, Russia, Spain, and Switzerland; 3 to Europe and worldwide, and specifically to the Czech Republic, Japan, Portugal, and Thailand; two to Hungary, New Zealand, the Philippines, Poland, and Slovakia; and one to Bermuda, Brazil, China, Curacao, Estonia, Honduras, Hong Kong, Latvia, Luxembourg, Malaysia, Moldova, Namibia, Serbia, Singapore, Slovenia, and South Korea.

Importing or exporting semen to and from other countries was not done by 59 of the respondents and was reported by 135 of the respondents. When asked to and from which countries semen had been shipped, 31 reported shipment to or from the United Kingdom; 30 to or from Canada; 27 to or from the United States; 22 to or from Germany; 18 to or from Sweden; 17 to or from the Netherlands; 15 to or from Australia; 13 to or from Finland; 12 to or from Hungary, ten to or from France and Italy; nine to and from Denmark; six to and from New Zealand and Russia; five to and from Belgium, Japan, and Norway; three to and from Slovakia, Slovenia, Switzerland, and Thailand; two to and from Europe, and specifically to and from the Czech Republic, Israel, Poland, South Africa, Spain, and Ukraine; and one worldwide and more specifically, to and from Austria, Brazil, Croatia, Estonia, Ireland, Kenya, Korea, Latvia, Mexico, Moldova, Peru, Portugal, Romania, Serbia, Singapore, the United Arab Emirates, Zambia, and Zimbabwe.

Diet provided for adult, non-pregnant breeding dogs was a dry, commercial diet in most of the facilities (Table 2). Brand or kind of food provided was Purina in 19 facilities; Fromm in 12; Eukanuba in nine; Royal Canin in eight; Kirkland in six; Orijen or Taste of the Wild in four; 4Health, Innova, Natural Balance, Nature's Variety, or NutriSource in three; Acana, Blue Seal, Diamond, Evanger's,

Hill's, Honest Kitchen, Life's Abundance, or Merrick in two; and in one facility each, American National, Annamaet, BilJac, Black Gold, Blue Buffalo, Bosch, Canidae, Earth Borne, Evo, Evolve, Flint River Ranch, Holistic Select, Native, Pedigree, Pinnacle, Salters, Sojo's, Sports Mix, Tractor Supply, and Wainwright's. Minimum requirements for nutrition by life stage have been described for dogs but there is little in the literature concerning optimal diet for breeding dogs.[3,4]

Disease control and incidence

Figure 2 and Table 3 show information regarding for which diseases adult dogs are vaccinated in these facilities and with what frequency. Figure 3 demonstrates which diseases or conditions have been identified by breeders in these facilities. Frequency of vaccination and use of antibody titers to identify disease risk are current research and discussion topics in veterinary medicine. Guidelines of the American Animal Hospital Association (AAHA) recommend that adult dogs be vaccinated against canine distemper, infectious canine hepatitis, canine parvovirus and parainfluenza virus at least every three years after appropriate vaccination as a puppy, and state that protective titers are expected to be maintained in healthy dogs for at least 5 years.[5] Vaccines for *Bordetella bronchiseptica*, leptospirosis, Lyme disease, and coronavirus are non-core vaccines. Veterinarians and dog owners are expected to follow local laws regarding use of rabies vaccines. Bitches should be vaccinated well ahead of expected onset of estrus, to ensure high titers during pregnancy and formation of colostrum; vaccination during pregnancy is not recommended. Vaccination against canine herpesvirus is not available in the United States but is available in Europe; this vaccine is given just prior to or during estrus and pregnancy.[6,7]

When asked about availability of a veterinarian licensed as a specialist in reproduction, either through the ACT or European College of Animal Reproduction, 305 respondents stated that they did have access to such a veterinarian and 16 (34.7%) responded that they did not. This clearly is an opportunity for veterinary medicine, as one-third of those surveyed were underserved by our profession in this regard.

Nineteen respondents reported never testing their dogs for canine brucellosis. Two hundred four reported testing for brucellosis, with the majority (n=159) testing before breeding; 16 testing every 12 months; ten testing every six months; five testing prior to semen collection and/or shipment; three testing when first purchased, when they enter the kennel, or prior to semen freezing; and one each testing when reach breeding age, every 6-12 months, greater than every 12 months, after whelping, or variably. Brucellosis is not a disease seen world-wide so it is not surprising that not all breeders in this survey test for the disease. However, breeders have been identified as a group at risk for exposure and are not routinely identified as having knowledge of the disease.[8] Veterinary recommendations regarding frequency of testing must vary with type of test used. In a survey of members of the ACT and SFT, the majority recommended that bitches be tested at the time of breeding and males be tested at the time of breeding or semen collection, or twice yearly.[2]

Management of breeding dogs

When asked about genetic testing, only one respondent reported never having dogs tested for hereditary conditions. One hundred eighty nine respondents reported such testing (Table 4). The members of the ACT and SFT strongly recommend that only genetically superior, healthy dogs be used for breeding. All breeds recognized by the American Kennel Club (AKC) have a national parent club with a health committee and specifics regarding testing for hereditary disease can be found on the parent club website, available through the AKC website (www.akc.org). When asked specifically about testing for hip dysplasia, 86 respondents reported using the Orthopedic Foundation for Animals (OFA) only, 50 reported using both OFA and PennHIP, and five reported using PennHIP only. Some reported using OFA for one breed and PennHIP for another, depending on how large the database was in a given system for their breed. This suggests a vicious cycle may exist in some breeds, where breeders do not test using a given message because the database is small, keeping that database small through lack of testing. Eleven respondents submitted films to the British Veterinary Association; ten to authorized or experienced veterinarians; four to the Swedish Kennel Club or an association using the classification scheme of the

Federation Cynologique Internationale; three to the Australian Veterinary Association; two to the Norwegian Kennel Club; and one each to the Finnish Kennel Club and the Netherlands Kennel Club.

Litters per year produced was fewer than 1 for 90 respondents, 1 for 48 respondents, 2 for 24 respondents, and 3 for 12 respondents. Three people responded that they produced 4 litters per year and 4 that they produced 5 litters per year. Others reported producing 7, 8, 10, 12-15, 14, 20, and 60 litters per year. Litters produced from a given male, on average, was 1 in 26 facilities, 2 in 14, 3 in 23, 4 in 13, 5 in 17, 6 in 8, 7 in 6, 8 or 9 in 4, and 10 in 7, with others reporting production of anywhere from 12 to more than 50 litters produced from a given male in his lifetime. Litters produced from a given bitch, on average, was 1 in 35 facilities, 2 in 70, 3 in 42, 4 in 21, 5 in 10, and 6 in 3 facilities. No one reported more than 6 litters from a given bitch. The surveyed veterinarians in the ACT and SFT suggested 2 to 3 litters as a maximum number in a bitch's lifetime, with that recommendation largely based on experience.[2] Reports of maximal breeding productivity in large commercial kennels have identified five litters as the maximum number for bitches.[9,10]

The youngest age at which a bitch is used for breeding was overwhelmingly reported as 2 years of age (n=104). Others responded with 12 to 18 months of age (n=7), 18 to 24 months of age (n=29), 2.5 years of age (n=18), 3 years of age (n=27), 3.5 years of age (n=1), 4 years of age (n=7), or 5 years of age (n=1). Some reported that the earliest a bitch would be bred was her second heat (n=1) or third heat (n=2). Sexual maturity in bitches is defined as onset of the first proestrus, and physical maturity, defined by closure of physes in the long bones, happens at about this same time.[11] Behavioral maturity is reported to occur at an age approximately twice that of sexual maturity.[12] Veterinarians in the ACT and SFT showed similar variation in recommended age at first breeding, with most recommending an age beyond that at which testing for hereditary disease could be completed.[2]

The oldest age at which a bitch is used for breeding was 6 to 7 years, with 1 respondent stopping breeding at 3 years of age, 8 at 4 years of age, 32 at 5 years of age, 1 at 5.5 years of age, 53 at 6 years of age, 4 at 6.5 years of age, and 43 at 7 years of age. Others reported using bitches until up to 10 years of age, with the majority of those using bitches until 8 to 9 years of age. Three people reported reliance on veterinary advice and 14 respondents stated that it depended on the health and mothering ability of the bitch and quality of the pups produced. Reported health risks in older bitches when bred include decreased conception rate, and decreased litter size with coincident increase in puppy size and predisposition to dystocia.[2] These risks vary by breed, with giant breeds showing such complications as young as five years of age and toy breeds perhaps not showing such complications until 10 years of age or older.[13]

When asked how frequently bitches are bred, the majority of participants reported that bitches are bred no more than once per year (Table 5). Frequency of breeding, especially considering back-to-back breeding, is a common concern raised by breed organizations. There is no published science to guide this decision. Over 50% of the veterinarians from the ACT and SFT who were surveyed believed that bitches could be bred every cycle as long as the bitch and pups were healthy and the bitch had regained body condition after lactation.[2] Breed club requirements may limit frequency of breeding by limiting number of litters that can be registered to a given bitch annually.

One hundred twenty-five respondents had used fresh semen for artificial insemination (AI), while 91 had used chilled semen, and 71 had used frozen semen. Eighty-seven respondents reported using only natural service. Fresh semen was overwhelmingly the type of semen most commonly used by participants who had used more than one type of semen. One respondent reported that AI is not permitted in dogs to be registered in their country of residence. Respondents using fresh semen most commonly used vaginal insemination (n=115), with transcervical and surgical insemination used less commonly (n=13 and 10, respectively). Respondents using chilled semen also most commonly used vaginal insemination (n=112), with a fairly large number using transcervical insemination (n=71) and a lower number using surgical insemination (n=41). Respondents using frozen semen most commonly used surgical insemination (n=135), with some using transcervical insemination (n=37) and a very small number using vaginal insemination (n=5). Those respondents who had had semen frozen from their dogs most commonly had

done so through private veterinary clinics (n=187) with some using lay personnel (n=21) and some using academic institutions (n=15).

Participants were asked if they treated their bitches for internal or external parasites during heat and pregnancy. Forty-four respondents did not treat bitches during heat or pregnancy. Six treated during heat but not during pregnancy, and 7 treated only if the bitch was diagnosed with worms or the veterinarian specifically recommended treatment. One hundred forty-one respondents reported treating bitches during heat or pregnancy (Table 6).

Management of whelping and puppies

Average litter size in the 191 facilities for which responses were given varied from 1 to 11 with a mean of 6.3 and a median of 5.0. Incidence of dystocia or request from a veterinarian for assistance during whelping varied from never to always (Figure 4). Nine respondents always scheduled cesarean sections. Participants described specific things done to minimize risk of dystocia in their facilities including breeding to an appropriately sized male, exercising the bitch appropriately during pregnancy, not misusing calcium supplements during pregnancy, being patient during whelping, and recognizing breed differences.

When asked at what environmental temperature puppies are housed in the first weeks of life, 18 respondents described it as room temperature. Most respondents maintained puppies between 71 and 80°F (Figure 5). This is lower than the recommendation commonly cited in veterinary texts.[14] Supplemental heat sources supplied were heating pads or mats, or electric blankets (n=110); heat lamps (n=55), space heaters in the room (n=21), heating discs (n=13), a heated floor surface (n=9), rugs or blankets over the whelping box or pen (n=7), hot water bottles (n=6), and a heated bed or heating stones (n=1 each). Twenty-six respondents reported using no external heat and many comments on how this varied with time of year and location of the facility.

Puppies were first introduced to solid food and weaning begun at 3 to 4 weeks of age in most facilities (range = 1.5 – 7 weeks, mean = 3.0 weeks, median = 4.0 weeks). Six participants reported letting the bitch decide on weaning timing, and one responded that weaning began when the puppies' teeth began to erupt. Food offered to begin weaning was puppy kibble moistened with water, goat's milk, yogurt, or milk replacer (n=83); raw food (n=34), human dairy, baby cereal or cooked meat (Gerber, baby food, yogurt) (n=27); dry puppy kibble (n=10), Royal Canin starter (n=7); and canned puppy food, Eukanuaba weaning formula, goat's milk, or a homemade weaning formula (n=2 each). Breeders should be educated in the relative lack of nutritional value in human foods and goat's milk and encouraged to use foods more appropriate for dogs at this life stage. Weaning was completed by 6 to 8 weeks of age in most facilities (range = 3.5 to 12 weeks, mean = 6.5 weeks, median = 7.0 weeks). Fifty participants reported letting the bitch decide when weaning was complete.

Puppies were evaluated by a veterinarian before being placed in 218 facilities. Two respondents did not have pups evaluated by a veterinarian before placement. Twelve only had a veterinary health check if required for shipment. Sixteen respondents took the pups to the veterinarian just before placement, and fifteen did not provide a specific age when such checks were performed. Of those respondents giving a specific age for evaluation, range of age was 1 to 16 weeks, with a mean of 7.6 weeks and a median of 7.0 weeks. Most puppies were seen by a veterinarian just once before placement (n=128), with 26 seen two times, 6 three times, and 4 four times before placement; presumably this varied with vaccination schedule and with availability and appropriateness of specialty examinations (for example, Baer hearing testing, cardiac evaluation).

Vaccination of puppies for common diseases is shown in Figure 6. Twenty-nine respondents stated that they vaccinated but with no further information. Five respondents did no vaccinations of pups before placement. Five respondents followed guidelines espoused by Dr. Jean Dodds, and 1 each responded that they followed the law, AAHA guidelines, or World Small Animal Veterinary Association (WSAVA) guidelines. Twenty-three respondents listed age at first vaccination without stating which vaccines were given; range was less than 4 weeks to 10 weeks of age, with a mean of 7.5 weeks and median of 9 weeks. Rabies vaccinations were given as required by law. American Animal Hospital

Association guidelines call for vaccination for canine distemper, infectious canine hepatitis, canine parvovirus, and parainfluenza every 3 to 4 weeks between the ages of 6 and 16 weeks and vaccinating for rabies as required by law.[5]

Deworming of puppies is shown in Figure 7. Eight respondents stated that they never deworm puppies before placement. Nine respondents stated that they do deworm pups but gave no more information. Twenty-five respondents deworm puppies only if a fecal flotation or direct fecal examination identifies infection or on a veterinarian's advice.

Puppies are placed in new homes by 8-9 weeks of age in most facilities (range 4 - 18 weeks, mean = 9.9 weeks, median = 9.0 weeks). Other responses included that timing was dependent on their potential value as a show, working, or breeding prospect (n=4); on the puppy's maturity or on the home they're going to (n=3 each); or on their size (n=2). One respondent kept puppies until 1.5 years of age and two respondents kept puppies until the right home was available. When asked how suitable homes are chosen, 105 respondents cited a mandatory interview, either by email or telephone or in-person; 85 reported use of an application or questionnaire; 72 required an in-person meeting; 39 required a referral of some sort, including direct referral, word of mouth, and use of social networking tools; 37 reported requiring check of the potential home, either by an in-person visit or through use of images; 34 required and checked letters or other testimonials of referral; 17 require a recommendation from the potential owner's veterinarian; 5 used a written contract; 4 did an Internet check of potential owners and their homes; 3 required a background check, and 1 respondent each required an introductory letter written by the potential owner or spoke to neighbors of the potential owner. The list of questions on applications or questionnaires that were described included questions to permit matching of the dog's temperament to the individual or family (lifestyle, make-up of family by age, presence of absence of family members during the day, time availability), information about past dogs owned, knowledge of the breed, plans for training and boarding the dog, plans for showing or working the dog, ability to afford a dog, okay from landlord if renting, willingness to take advice from the breeder, and willingness to join a kennel club or association related to the breed.

When asked if puppies were spayed or castrated before placement, 175 respondents said no and 24 said yes. Reasons given for spay/castration before placement included owner's request, not placing dogs until older (6-8 months) for a variety of reasons, hereditary defects (for example, cryptorchidism), and waiting to place an adult or mature dog. When asked the recommended age for spay or castration as per a contract when the pet was placed, for bitches the recommended age for ovariohysterectomy ranged from 6-24 months, with a mean of 13.5 months and median of 18 months. For stud dogs, recommended age for castration ranged from 6-19 months, with a mean of 12.4 months and a median of 15 months. Eleven participants recommended bitches be spayed after their first heat, and 7 respondents recommended spay or castration after sexual and physical maturity. Two respondents stated that it was the new owner's responsibility to decide when to have the animal spayed or castrated. The relatively late age recommendation for ovariohysterectomy of bitches likely is a reflection of current research demonstrating detriments of ovariohysterectomy.[15] Breeders are encouraged to work with their veterinarian to understand current findings and how they relate to dogs of their breed.

Breeders generally preferred to maintain some control over breeding rights of the dogs produced. Twenty-one respondents stated that new owners could use the dogs for breeding with no restrictions. Eighty-seven respondents never permit breeding of dogs produced and as a means of controlling this, cite such things as making the date of sale the date of proof of spay/castration surgery, or otherwise holding papers or restricting ownership until specific conditions are met. Ninety-five respondents sometimes permit use of dogs for breeding. Examples of conditions when breeding might be permitted included placement of a potential show or working champion; breeding permitted after achievement of a title or certification (for example, therapy dog); breeding with the breeder's permission, which may or may not include co-ownership, choice of mate, and requirement to use the same breeding contract as the original breeder; breeding permitted after passage of all health and genetic clearances; demonstration that the new owner is willing to do it "right" as defined by the original breeder; and requirement that the purchased dog and all puppies be registered with specific kennel clubs or breed associations.

Conclusion

This is a review of common practices, not necessarily best practices, by dog breeders from around the world. Lessons can be learned by the breeders themselves, by veterinarians who work with these breeders, and by the veterinary profession. Breeders would benefit from greater knowledge of infectious disease, especially brucellosis, and better understanding of vaccines, including against which diseases puppies and adult dogs should be vaccinated and with what frequency and with the concept of herd immunity. Greater knowledge of principles of bioexclusion would benefit facilities as well. Continuing discussion regarding suitability of back-to-back breedings and consideration of maximum number of litters from a given bitch or sire would benefit breed clubs. Finally, breeders should consider nutrition, especially as they start to wean pups. Veterinarians working with breeders can assist them by keeping themselves current and educating clients on the above topics. It is valuable for these veterinarians to be aware of common practices by breeders and to understand the number of dogs, not just the number of breeding dogs, in a given facility, and how they are best managed. Finally, the veterinary profession should recognize the need for continuing research in housing and diet of breeding dogs, and the need for increase in availability of theriogenology training to ensure properly trained professionals are available to serve all dog breeders.

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Figure 1. Survey instrument made available electronically to dog breeders

Thank you for participating in this survey. Please respond only about your own facility and do not include information from animals you own or breed that are not housed at your facility.

GENERAL INFORMATION
Job responsibilities (veterinarian, veterinary technician / veterinary nurse, human health care professional, none of the above):
Country of residence:
Dog breed(s) maintained for breeding:
Description of facility (hobby breeder, commercial breeder, service dog organization, other):
Number and ages of intact female dogs on premises (from breeds above only, not pets):
Number and ages of spayed female dogs on premises (from breeds above only, not pets):
Number and ages of intact male dogs on premises (from breeds above only, not pets):
Number and ages of castrated male dogs on premises (from breeds above only, not pets):
Breeding dogs are primarily housed: [ ] inside [ ] outside [ ] equally inside and out
If dogs are housed in a kennel or cage, size of enclosure (please denote sq ft or sq m):
Are dogs usually housed as a group or individually?
Are outside dogs permitted into the facility? Is so, how are they managed (quarantined, housed separately, immediately placed with group)?
Do you travel with your dogs to other countries? If so, to which countries?
Do you import or export dogs or semen to other countries? If so, to which countries?
What diet do you provide for adult, non-pregnant breeding dogs?

INFECTIOUS DISEASE
For which diseases are your dogs vaccinated (canine distemper, infectious canine hepatitis, parvovirus, parainfluenza virus, Bordetella bronchiseptica, canine herpesvirus, canine coronavirus, Borrelia burgdorferei (Lyme disease), leptospirosis, rabies):
How frequently do adult dogs receive booster vaccinations?
Which of these conditions have you seen in your facility (canine distemper, infectious canine hepatitis, parvovirus, parainfluenza virus, Bordetella bronchiseptica, canine herpesvirus, canine coronavirus, Borrelia burgdorferei (Lyme disease), leptospirosis, rabies, pyometra, ovarian tumor, ovarian cyst, mammary tumor benign, mammary tumor malignant, benign prostatic hypertrophy, prostatitis, prostatic tumor, testicular tumor, cryptorchidism):

REPRODUCTIVE PERFORMANCE
Do you have available to you a veterinarian licensed as a specialist in reproduction?
Are your dogs tested for brucellosis? If so, how frequently?
Are your dogs tested for genetic diseases? If so, which conditions? For hip dysplasia, are your dogs evaluated through OFA, PennHip or another system (please describe)?
How many litters/year do you produce?
How many litters do you produce, on average, from a given male dog in your line?
How many litters do you produce, on average, from a given bitch in your line?
What is the youngest age at which bitches are bred?
What is the oldest age at which bitches are bred?
What is the timing used for breeding bitches (back-to-back, no more than once yearly, etc)?
What kind of semen have you used for breeding, and which kind do you use most frequently (fresh, chilled, frozen)?
What form(s) of insemination have you used for each kind of semen (natural breeding, vaginal insemination, intrauterine transcervical, intrauterine surgical)?
If you have had semen frozen from your male dog(s), where was it frozen (university, private veterinary clinic, lay person):
Do you treat your bitches for internal or external parasites during heat and pregnancy? If yes, what product(s) do you use?

What is the average litter size in your facility?
What is the incidence of dystocia / how frequently do you take the bitch to a veterinarian for assistance whelping?
At what environmental temperature are puppies housed in the first weeks of life?
What supplemental heat sources do you provide in the first weeks of life, if any?
If you provide supplemental food while puppies are nursing, what do you provide?
At what age are puppies first offered solid food, and what kind of food do you offer?
By what age are puppies weaned?
By what age are puppies placed in new homes?
How are suitable homes for puppies chosen?
Do you permit pet owners to breed the dogs they buy from you? If so, do you restrict this in any way?
Are puppies evaluated by a veterinarian before being placed? If so, at what age?
Are puppies vaccinated before being placed? If so, for what diseases and at what age?
Are puppies dewormed before being placed? If so, for what diseases and at what age?
Are puppies spayed / castrated before being placed? If so, at what age?

Figure 2. Frequency of vaccination for which adult dogs are vaccinated in survey population

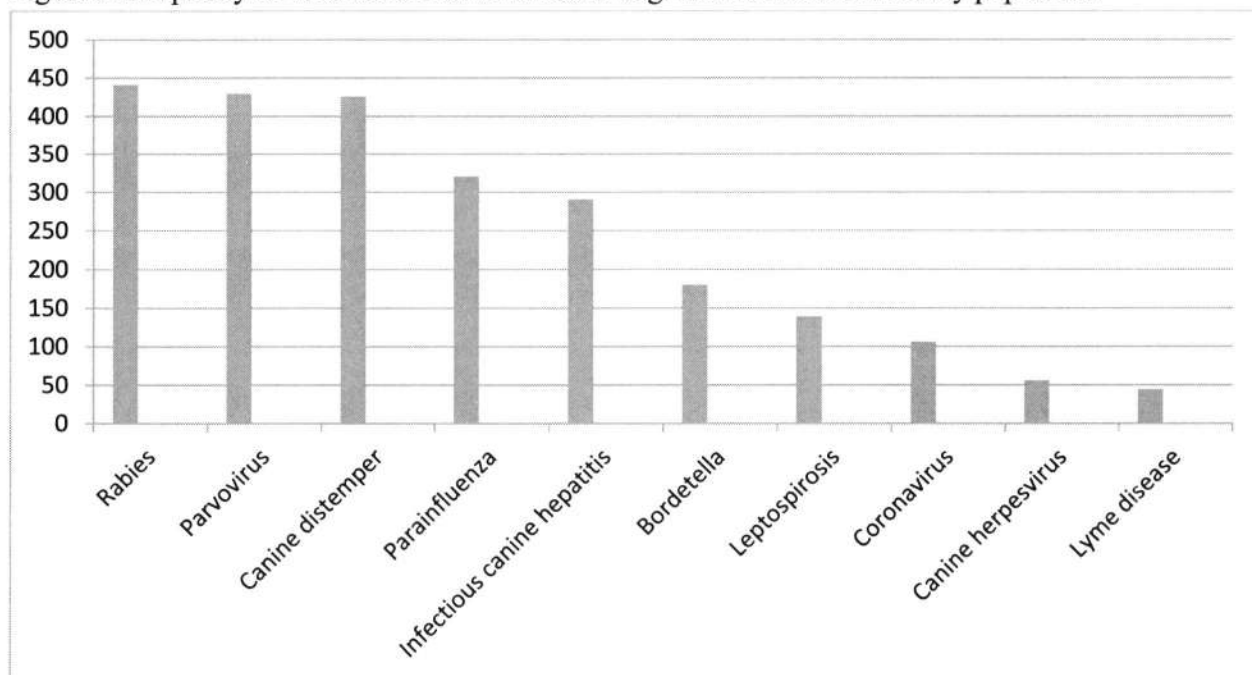


Figure 3. Frequency of infectious diseases and disorders seen in facilities in survey population

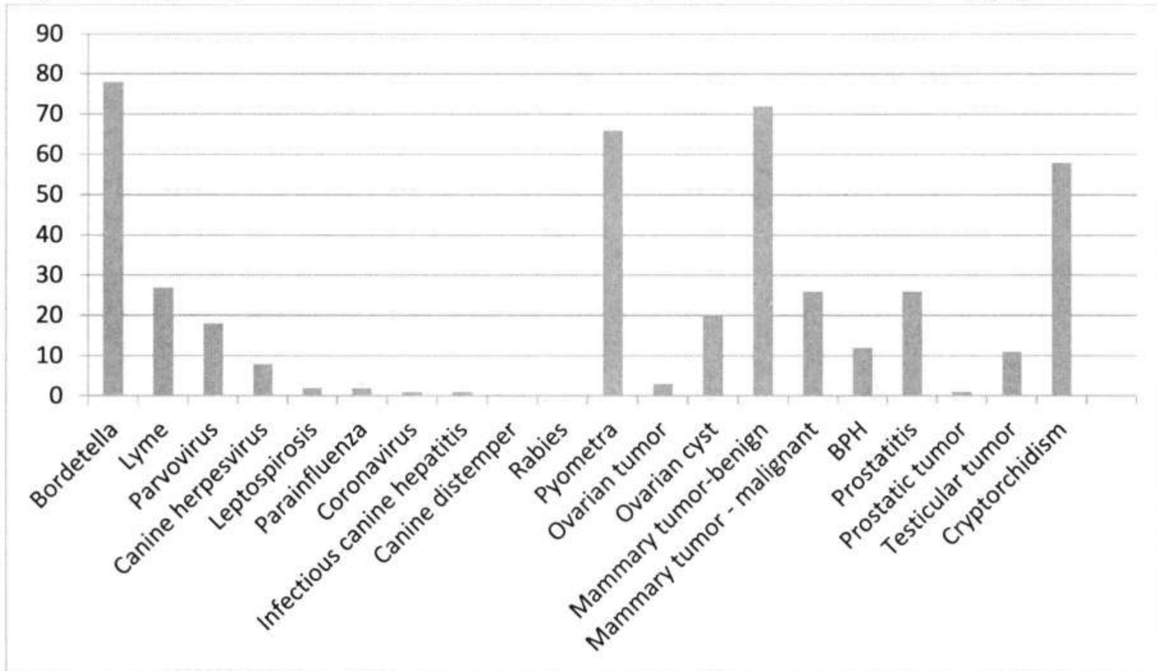


Figure 4. Incidence of dystocia in survey population

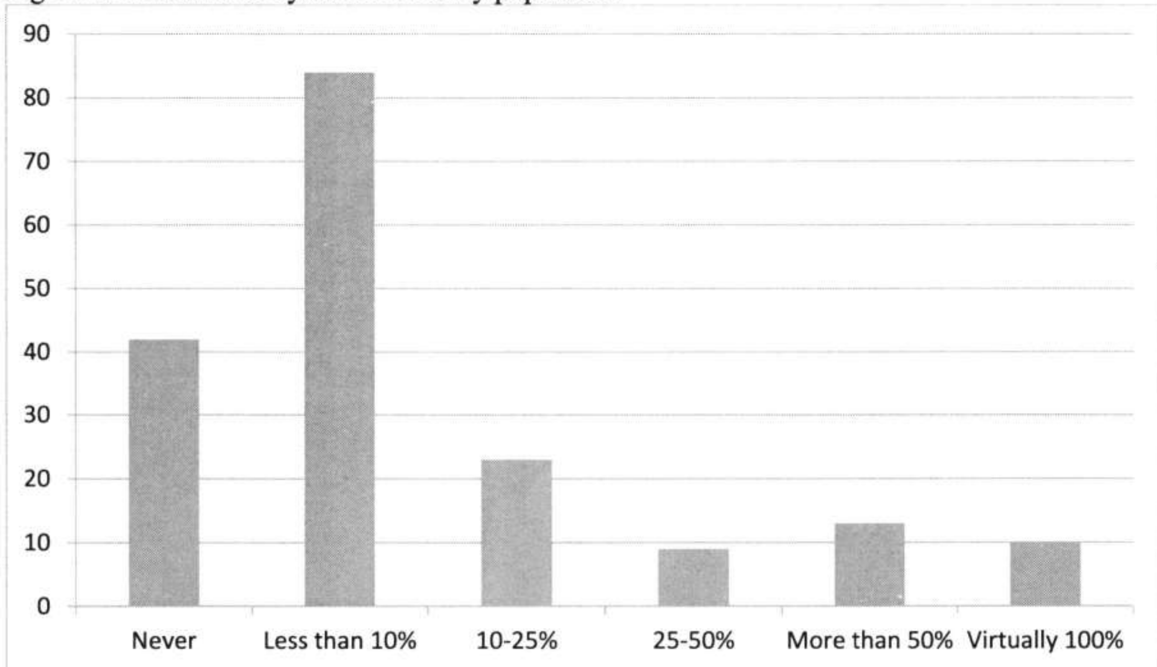


Figure 5. Environmental temperature at which puppies are maintained in first weeks of life

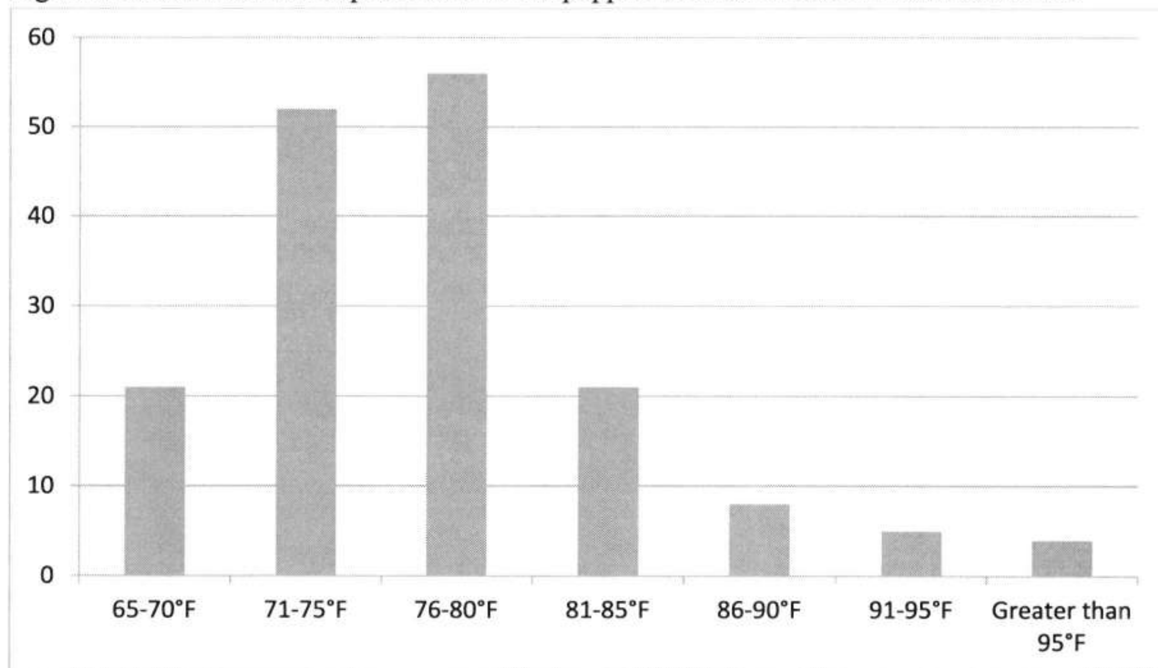


Figure 6. Vaccination type and frequency administered to puppies in survey population

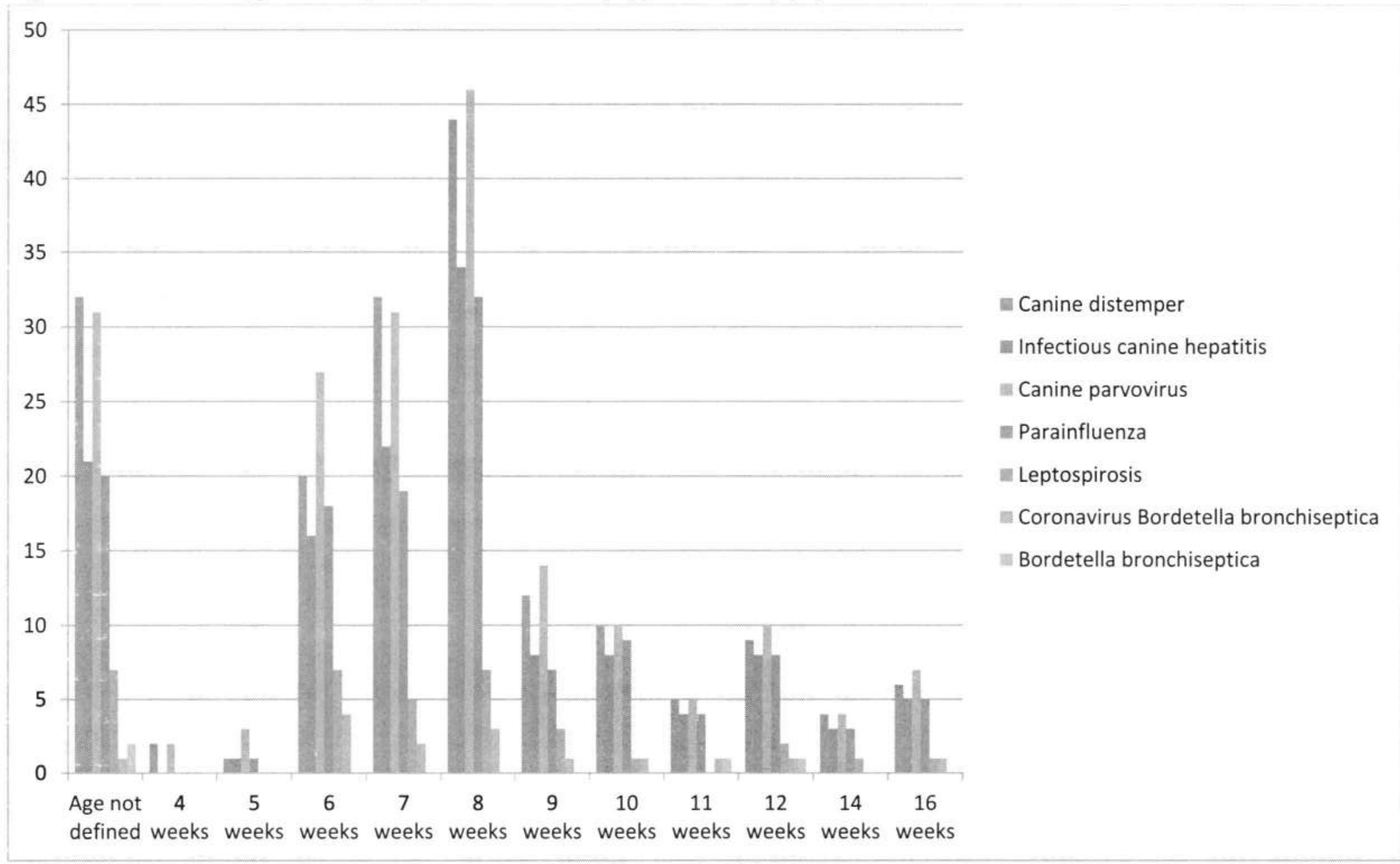
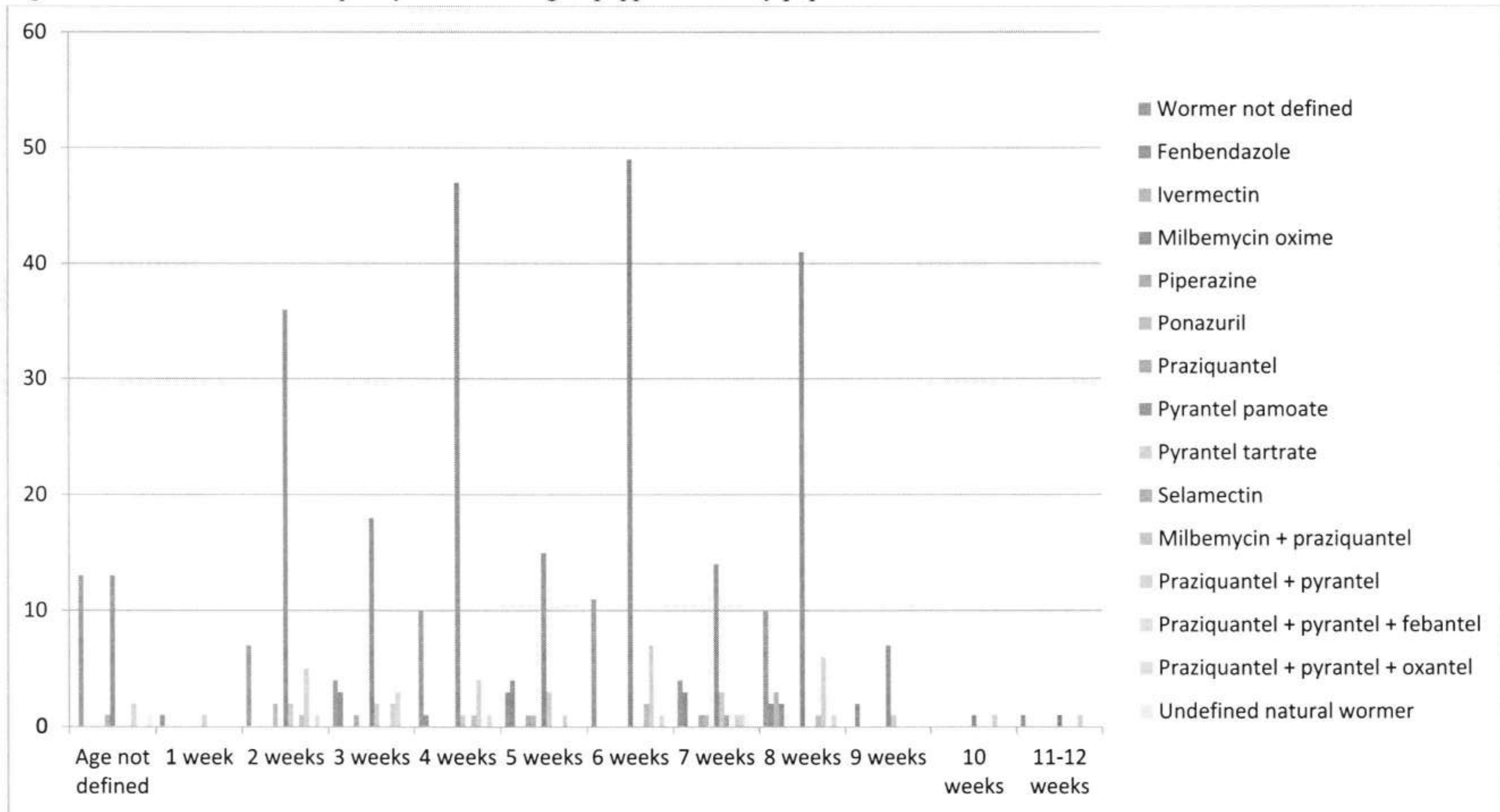


Figure 7. Products used and frequency of deworming of puppies in survey population



Brand names: Fenbendazole (Panacur, Safeguard); Ivermectin (Heartgard); Milbemycin oxime (Interceptor); Ponazuril (Marquis); Pyrantel pamoate (D-Worm, Evict, Nemex 2, Strongid); Pyrantel tartrate (Banminth); Milbemycin oxime + praziquantel (Milbemax); Praziquantel + pyrantel pamoate (Drontal, Pratel); Praziquantel + pyrantel pamoate + febantel (Dechinel+); Praziquantel + pyrantel pamoate + oxantel (Canex)

Table 1. Dog breeds represented in the survey population

BREED(S)	NUMBER OF FACILITIES
Labrador retriever	11
Australian shepherd	9
Shetland sheepdog	8
Golden retriever, Standard poodle	7
German shepherd dog	6
Bernese mountain dog, border collie, Cavalier King Charles spaniel, Irish wolfhound, Norwich terrier	5
Alaskan malamute, Beagle, Chihuahua, Dachshund, Miniature poodle, Rottweiler, Soft-coated wheaten terrier	4
Belgian malinois, Border terrier, Borzoi, Coton de Tulear, Doberman pinscher, English cocker spaniel, English setter, English springer spaniel, Havanese, Nova Scotia duck tolling retriever, Parson Russell terrier, Rhodesian ridgeback, Samoyed, Staffordshire bull terrier, Vizsla	3
American cocker spaniel, Boxer, Brittany spaniel, Brussels griffon, Chinese crested, Flat-coated retriever, French bulldog, German shorthaired pointer, Gordon setter, Great Dane, Great Pyrenees, Jack Russell terrier, Maltese, Maremma sheepdog, Mastiff, Miniature dachshund, Pembroke Welsh corgi, Pug, Rough collie, Shih tzu, Smooth collie, Thai ridgeback dog	2
Akita, American Eskimo dog, American pitbull terrier, American Staffordshire terrier, Australian kelpie, Basenji, Belgian shepherd, Belgian tervuren, Berger picard, Black and tan coonhound, Boston terrier, Briard, Bulldog, Cairn terrier, Canaan dog, Cane corso, Cardigan Welsh corgi, Central Asian shepherd dog, Chesapeake Bay retriever, Chow Chow, Dalmatian, Entlebucher mountain dog, Finnish lapphund, German pinscher, German wirehaired pointer, Giant schnauzer, Grandane, Greater Swiss mountain dog, Ibizan hound, Irish red and white setter, Irish setter, Kai Ken, Keeshond, Lagotto Romagnolo, Lhasa apso, Miniature pinscher, Norfolk terrier, Papillon, Pomeranian, Poodle, Portuguese podengo, Portuguese water dog, Puli, Pyrenean mountain dog, Pyrenean shepherd, Rat terrier, Schipperke, Scottish terrier, Shiba Inu, Shiloh shepherd, Siberian husky, Silken windhund, Smooth fox terrier, Swedish vallhund, Tibetan terrier, Toy fox terrier, Toy poodle, Weimeraner, Welsh springer spaniel, Whippet, Wirehaired dachshund, Yorkshire terrier	1

Table 2. Types of food provided for adult, non-pregnant breeding dogs

TYPE OF FOOD	NUMBER OF FACILITIES
Dry only	108
Raw only	29
Cooked only	1
Dry and raw	34
Dry and canned	11
Dry and cooked	8
Raw and cooked	1
Dry and canned and cooked	2
Dry and raw and cooked	2
Dry and canned and raw	1

Table 3. Frequency of vaccination of adult dogs in survey population

FREQUENCY OF VACCINATION	NUMBER OF FACILITIES
Never	11
Once after puppy shots	18
Every year for life	37
Every 2-3 years for life*	55
Greater than every 3 years for life	14
Dependent on antibody titers	39
Annually until dog mature	7
Every 2-3 years until dog mature	10
Only before breeding or travel	4
At interval recommended by veterinarian	2
Only when disease confirmed (local outbreak, new strain identified)	2

* 3 years more commonly reported than 2 years

Table 4. Frequency of testing for heritable disease in survey population

TYPE OF DISORDER / TEST	NUMBER OF FACILITIES
CARDIAC DISORDERS	
Heart testing in general	78
Arrythmogenic right ventricular cardiomyopathy	7
Tricuspid valve dysplasia	1
Mitral valve disease	1
OPHTHALMIC DISORDERS	
Canine eye registry foundation (CERF)	113
Progressive retinal atrophy	50
Hereditary cataracts	11
Primary lens luxation	10
Collie eye anomaly	9
Glaucoma	5
Retinal dysplasia	5
Heat shock transcription factor mutation	4
Juvenile cataracts	2
Multifocal retinopathy	2
Progressive rod-cone degeneration	2
Australian canine eye scheme (ACES)	1
Cone-rod dysplasia 1	1
Congenital stationary night blindness	1
Keratoconjunctivitis sicca	1
HEMATOLOGIC DISORDERS	
VonWillebrand's disorder	22
Pelger Huet anomaly	3
Trapped neutrophil syndrome	3
Factor VII deficiency	3
Dog leukocyte antigen	2
Canine leukocyte adhesion deficiency	1
MULTISYSTEMIC OR METABOLIC DISORDERS	
Thyroid testing	58
Juvenile Addison's disease	4
Protein-losing enteropathy or nephropathy	4
Familial nephropathy	3
Liver shunt	2
Cystinuria	1
Fanconi syndrome	1
GM1 storage disease	1
Hyperuricosuria	1
Juvenile renal disease	1
Major histocompatability complex	1
Primary hyperparathyroidism	1
Renal disease	1
Trypsin-like immunoreactivity	1
NEUROMUSCULAR OR MUSCULOSKELETAL DISORDERS	
Hip dysplasia	139
Elbow dysplasia	79
Degenerative myelopathy	35
Patellar luxation	35
Exercise-induced collapse	10

Centronuclear myopathy	7
Neuronal ceroid lipofuscinosis	5
Spinocerebellar ataxia	4
Legg Calves Perthes	3
Osteochondritis dissecans	3
Episodic falling	2
Late onset ataxia	2
Osculoskeletal dysplasia	2
Spondylosis	2
Bandera's neonatal ataxia	1
Benign juvenile epilepsy	1
Caudal occipital malformation syndrome	1
Cerebellar degeneration	1
Epilepsy	1
L-2-hydroxyglutaric aciduria	1
Lumbosacral transitional vertebrae	1
Muscular dystrophy	1
Neonatal encephalopathy	1
Neonatal ataxia	1
Stifle arthrosis	1
Syringomyelia	1
MISCELLANEOUS DISORDERS OR CONCERNS	
Multi-drug resistance 1	20
Baer hearing testing	13
Coat	10
Cleft palate 1	4
Ichthyosis	4
Bite / dentition	2
Familial enamel hypoplasia	1
High toe	1

Table 5. Timing used for breeding bitches

FREQUENCY OF BREEDING	NUMBER OF FACILITIES
Back-to-back, then skip a season	38
No more than every 6 months	6
No more than once per year	54
No more than once every 2 years	15
Every other season	11
No more than every third season	1
Dependent on club regulations or rules	4
Dependent on size of litter, quality of litter, bitch health, length of interestrous interval	37
Dependent on when breeder is ready to produce a litter	11
Dependent on veterinarian recommendation	4

Table 6. Parasiticides used during heat and pregnancy in survey population

CHEMICAL(S)	BRAND NAME(S) REPORTED	PARASITES TREATED	NUMBER OF FACILITIES
Fenbendazole	Panacur, Safeguard	Roundworms, hookworms, whipworms, some tapeworms	35
Fipronil	Fiprostar	Fleas and ticks	1
Imidacloprid	Advantage	Fleas	10
Ivermectin		Heartworm preventative	23
Milbemycin oxime	Interceptor	Roundworms, hookworms, heartworm preventative	9
Moxidectin	Proheart 6	Heartworm preventative	1
Nitenpyram	Capstar	Fleas	1
Permethrin		Fleas	1
Praziquantel	Biltricide	Tapeworms	1
Pyrantel pamoate	Nemex, Strongid	Roundworms, hookworms	19
Selamectin	Revolution	Heartworm preventative	5
Spinosad		Fleas	1
Etofenprox and s-methoprene and piperonyl butoxide	Biospot	Fleas and ticks	1
Fipronel and cyphenothrin	Sentry Fiproguard Max	Fleas and ticks	1
Fipronel and s-methoprene	Frontline +	Fleas and ticks	25
Imidacloprid and permethrin	K9 Advantix	Fleas and ticks, repels mosquitoes	4
Imidacloprid and pyriproxyfen	Advantage II	Fleas	1
Ivermectin and praziquantel	Heartgard +, Triheart	Roundworms, hookworms, heartworm preventative	30
Milbemycin oxime and lufenuron	Sentinel	Roundworms, hookworms, whipworms, fleas, heartworm preventative	8
Milbemycin oxime and praziquantel	Milbemax	Roundworms, hookworms, whipworms, tapeworms, heartworm preventative	7
Praziquantel and pyrantel pamoate	Drontal	Roundworms, hookworms, tapeworms	9
Spinosad and milbemycin oxime	Trifexis	Fleas, heartworm preventative	3
Heartworm preventative (undefined)			8
Ilium brand parasiticides			1
Apple cider vinegar			2
Cedar oil			1
Diatomaceous earth			11
Essential oils			1
Garlic			3
Virgin coconut oil			2

(Editor's note: The graphs in this publication are available in color in the on-line version of Clinical Theriogenology.)

Imaging of the small animal female reproductive system

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Abstract

The normal non-gravid uterus and ovaries of dogs and cats are either not seen or are poorly visualized with radiography. These organs are more clearly seen when enlarged because of pregnancy or disease. Ultrasonography allows pregnancy diagnosis at an earlier gestational age and allows better characterization of diseased organs. This imaging modality can serve as a diagnostic tool for conditions such as pyometra, endometritis, dystocia, failure of involution, ovarian cysts, and uterine or ovarian neoplasms.

Keywords: Pregnancy diagnosis, ultrasonography, radiology, pyometra, failure of involution

Normal reproductive tract

The normal non-gravid uterus is generally not distinguishable from the small intestine on radiographs of dogs. The ovaries are also not normally seen radiographically. In cats, the bladder is located more cranially than in dogs allowing the uterus to be occasionally visible. In a study of 50 intact cats examined with digital radiography, the uterus could be identified as a longitudinal fluid-opaque structure between the bladder/urethra located ventrally and the colon located dorsally in 14 cats. The uterus was best seen when compression was applied to the caudal abdomen with a wooden spoon. Iodinated contrast can be placed in the vagina using a Foley catheter to check for fistulae or tears but in most cases, ultrasonography has more value for imaging the female reproductive tract.

A high frequency transducer (7-12 MHz) should be used for all cats and most medium sized dogs although a 5 MHz transducer can be used for very obese or large dogs. It is best to avoid scanning the uterus after an enema, which would increase colonic gas. Sometimes a partially distended urinary bladder can serve as sonic "window." The patient can be scanned in either lateral or dorsal recumbency. In dorsal recumbency, the uterus is more likely to be symmetrically placed and is easier to follow but it can sometimes be easier to identify the uterus from the lateral aspect where it is more superficial. If the ovary can be located, scanning can be done between the ovary and bladder to detect the uterus although the uterus is smaller close to the ovary. The ovaries are best examined with the patient in left and right lateral recumbency since they are dorsally located near the kidneys. There is greater difficulty if the probe is placed ventrally with the patient in dorsal recumbency because there will be greater distance between the transducer and ovary and there will be more intervening loops of bowel containing gas. If the probe is placed longitudinally on the body wall, the ovary can be found superficially deep to the skin and peritoneum, usually within 2 cm ventral, caudal, caudolateral, or caudomedial to the kidney. In a few greyhounds we imaged, the ovary was found medial to the mid-portion of the kidney at ovariohysterectomy.

In the non-gravid female, the uterus is best seen when the patient is in estrus. In the transverse plane, the uterus appears as a homogeneous hypoechoic circular structure or ellipse, about 0.5-1.0 cm depending on body weight. Usually the myometrium and endometrium cannot be distinguished. In the longitudinal plane, the uterus appears as a solid, slightly undulant, tubular structure. The body and horns are homogeneously echoic with no apparent lumen except during estrus when some blood may normally be present. During estrus and early pregnancy, the horns become mildly enlarged and more hypoechoic; some fluid may be in the lumen. An endometrial stripe is occasionally seen.

Care must be taken to avoid mistaking the aorta, caudal vena cava or small intestine for the uterus. The aorta and caudal vena cava can be distinguished from the uterus by using Doppler ultrasonography, if available. Otherwise, differentiation can be made because vessels are usually anechoic with a proper gain setting so that they appear as tubular structures with echogenic parallel walls in the sagittal plane. Additionally, pulsatility may be noted in the aorta and both vessels branch caudally whereas the uterus bifurcates cranially. Unlike the small intestine, the uterine wall does not exhibit wall

layers. Peristalsis and hyperechoic gas or ingesta can usually be seen in the intestinal lumen. Differentiation is more difficult if infiltrative disease in the small intestine causes a loss of wall layering. In that case, following the abnormal area cranially and caudally should reveal a connection to normal bowel.

The normal cervix is usually dorsal and slightly cranial to the trigone of the urinary bladder. In transverse images, the normal cervix appears as a hyperechoic, concentric structure, most prominent in dogs during proestrus, estrus, and early pregnancy. During estrus, the cervix is enlarged and hypoechoic, with a central hyperechoic ring. In the immature dog and during late pregnancy, the cervix is flatter and more difficult to identify.

The normal vagina is often found within pelvic canal where it is difficult to see transabdominally because of overlying bony structures. When visualized, hyperechoic folds may be seen centrally and the uterus may be triangular in cross-section. In estrus, the vagina is more hypoechoic.

The normal ovary is approximately 1.5 cm x .7 cm x .5 cm in 25 lb dog. The feline ovary is usually less than 1 cm in length. Follicles may be visible in the outer cortex while the medulla is located centrally. In estrus, the ovaries become larger, rounder, and contain mature follicles. Size increases with follicular development. Ovulation is signaled by a decrease in follicular size and number, which can fall to zero. Although it is tempting to think that sonography could be used to determine the time of ovulation, this is usually not possible because it is difficult to differentiate between a corpus luteum and follicle.

Follicles appear as anechoic or hypoechoic cyst-like structures and are first seen in proestrus, increasing in size until ovulation when they reach a maximum size of 1 cm in larger dogs. Some do not rupture and may be seen up to 7-9 days after ovulation in the gravid animal or may remain even longer in the non-gravid animal. After ovulation, the follicle fills with blood (corpus hemorrhagicum). Then, the blood is resorbed (corpus luteum). The corpus luteum persists through pregnancy or diestrus (if pregnancy does not occur). The corpus luteum degenerates into area of scar tissue (corpus albicans) when the animal delivers. Large corpora lutea appear as circular hypoechoic regions that may contain fluid and that resemble follicles. They may be solid, deforming the ovarian surface, and may have a thick wall. Hyperechoic areas in the ovary can represent fibrovascular connective tissue. Gradually fluid-filled corpus lutea decrease in size and become more echogenic until in anestrus or diestrus, the ovary is difficult to find.

Pregnancy

On radiographs, mineralization of the fetus is visible at about 45 days post LH surge in the dog. In the cat, uterine enlargement is present at 25-35 days of gestation. Mineralization of the fetal skeleton is present at 26-45 days of gestation.

Using sonography, pregnancy can be detected in dogs as early as 17-20 days after the luteinizing hormone peak (15-20 days after 1st breeding). Initially, the chorionic cavity will be seen as a 1-2 mm anechoic spherical vesicle. The embryonic mass appears about 5 days later at the periphery of the chorionic cavity. The fetal heartbeat becomes apparent. Pregnancy diagnosis is recommended at 27 days after the luteinizing hormone peak when the gestational sac is about 1.0 cm and there is a detectable heartbeat.

In the cat, the gestational sac can be detected on sonography at 11-14 days after breeding. The fetal pole shows as "echogenic linear density" at 15-17 days. Pregnancy diagnosis is recommended at 16-20 days. Note that for both dogs and cats, the accuracy of diagnosis is dependent on the resolution of the machine, imaging characteristics of the patient, and the experience of the sonographer.

Fetal age can be estimated based on the time of detection of structures. For example, the bladder and stomach are visible by 35-39 days after the luteinizing hormone surge in dogs and by 29-32 days after breeding in cats. Alternatively, calculations of fetal age or days to parturition can be made based on gestational sac diameter, crown-rump length, head diameter and body diameter. Prediction of fetal number is inaccurate as a fetus could be missed or counted more than once, intestinal gas could hide a

fetus, the gestational sac may be too small to see, and fetal death and resorption could occur at a later date.

The normal fetal heart rate is approximately 2x the heart rate of the mother. For dogs, the heart rate is 170-230 bpm while the rate is 200-220 bpm in cats. Bradycardia indicates hypoxia and fetal distress. For both species, heart rate < 170 signals fetal distress and heart rate < 150 signals an emergency situation.

In the postpartum female, the uterus is large, the walls are thick, moderately irregular, and moderately echogenic. The endometrium is the thickest layer initially. It is hypoechoic at placentation sites and hyperechoic in inter-placentation zones. Placentation sites are about 2.2-2.8 cm diameter while interplacentation zones are 1.0-1.5 cm in the early postpartum period. The myometrium initially has three layers: an inner hypoechoic circular muscle layer, a central hyperechoic fibrovascular layer, and an outer hypoechoic longitudinal muscle layer. The contents of the uterus are of mixed echogenicity including hypoechoic luminal fluid, more echogenic blood clots, and moderately echogenic fetal and maternal membrane remnants. Considerable involution occurs during the next three-24 days. The uterine wall gradually becomes thinner and less irregular. The endometrium is a moderately echogenic ring (thicker and more irregular at placentation sites. The myometrium forms a hypoechoic ring. The contents become more homogeneous and hypoechoic so that the uterus has a target-like appearance in transverse images. In a study of beagle dogs, involution was not complete until about 15 weeks. In a study on cats, noticeable layers were gone by 28 days with the uterus appearing as a hypoechoic tubular structure sonographically.

Abdominal masses

Differentials for a mid-abdominal mass seen radiographically include the spleen, a pedunculated mass from the liver, an enlarged lymph node, an intestinal mass, or an ovary or retained testicle. Renal masses will be in a dorsal location initially but can grow ventrally to fill the abdomen. When the uterus enlarges, it usually forms tortuous, tubular structures although focal enlargement can occur mimicking a neoplastic mass. The urinary bladder is caudal but may reach the central area if overly full or displaced. Ovarian masses may be found dorsally but frequently they are located ventrally because of stretching of the ovarian ligaments. On sonography, an ovarian mass may appear as a well-defined homogenous mass caudal and separate from the kidney. Anechoic areas can indicate cystic or necrotic areas. Hyperechoic areas with deep acoustic shadowing can indicate areas of mineralization. Possible masses include sex-cord stromal tumors, adenocarcinomas, and teratomas.

A right ovarian mass may displace the descending duodenum and ascending colon medially while a left ovarian mass may push the descending colon and adjacent small intestine similarly. A large mass may pull the ipsilateral kidney ventrally. Some ovarian masses can be dorsal and bilateral.

Dorsocaudal masses can be important in that they may indicate enlargement of the medial iliac lymph nodes. These nodes drain the hindlimbs and pelvis. Inflammatory or neoplastic change can be seen associated with involvement of the reproductive organs. Masses in the ventrocaudal region could be associated with the uterus or male organs. Enlargement of the uterus in this area is most often caused by localized pyometra, other fluid, or pregnancy. Neoplastic uterine masses are rare although leiomyomas, leiomyosarcomas, and adenocarcinomas have been reported.

Pyometra

On radiography, pyometra most commonly presents as tortuous tubular opacities in caudoventral abdomen. The uterus must be larger than the small intestine to be identified. On the lateral projection, the small intestines are pushed dorsally and cranially and there may be more separation between colon and bladder than is normally seen. A wooden spoon or commercial paddle is useful to separate and compress adjacent organs and improve visualization of the uterus in less obvious cases. On the ventrodorsal view, it is more difficult to appreciate the tortuous fluid-opaque horns. As mentioned, pyometra can occasionally cause a focal enlargement rather than enlargement of both horns. Physical examination, blood tests, and ultrasound can be used to differentiate causes of uterine enlargement.

Differentials include pregnancy and other causes of fluid in the uterus such as mucometra and hydrometra. On ultrasonography, the contents of the uterus will be anechoic or echogenic with a swirling pattern although the type of fluid cannot be determined sonographically. Occasionally, pyometra will occur in the stump remaining after ovariohysterectomy. The stump will be seen as a fluid-filled structure between the bladder and colon. The uterus should not be aspirated to avoid spilling purulent material into the abdomen.

If rupture of the uterus has occurred, the abdomen will exhibit a partial or complete loss of serosal detail on radiography. Other differentials include rupture of a hollow organ, ascites associated with cardiac or liver disease, hemorrhage, peritonitis, and carcinomatosis. On ultrasonography, the uterus will be distended with cellular appearing fluid. Similarly appearing cellular fluid will be seen in the peritoneal cavity.

Endometritis/cystic endometrial hyperplasia

Endometritis or hyperplasia can result in an irregularly thickened uterine wall. The wall may be thickened and may contain small cysts. A small amount of hypoechoic or anechoic fluid may be present in the lumen. Follicles or cysts may be noted in the ovaries.

Dystocia

On radiography, the size of the fetus should be evaluated in comparison to the pelvic canal. Radiography might also reveal healed fractures that could compromise the size of the pelvic canal. Radiographic signs of fetal death include loss of flexion, overlapping skull bones, and gas in the uterus, fetal thoracic or abdominal cavities, or fetal vessels. Loss of flexion by itself should be interpreted with caution. A fetus may momentarily be stretching.

Ultrasonography can be used to check for evidence of fetal distress signaled by a heart rate that is too fast or too slow. A heart rate <150 bpm equates to an emergency situation. If fetal death has occurred, there will be no detectable heartbeat or movement. Other sonographic evidence of fetal death includes excessive fluid in uterus, gas in the uterus or fetal tissues, and a loss of expected detail in the fetal anatomy.

In rare situations, a mummified fetus may be seen in the abdomen. If the fetus has been dead for some time, it may become a mineralized mass where the bones are compressed and the fetus is barely recognizable.

Failure of involution

The normal postpartum uterus is enlarged compared to the non-gravid appearance. The walls are thick, moderately irregular, and moderately echogenic. The myometrium and endometrium are distinguishable. Most involution should occur over about three-24 days in dogs but the involution is not complete until about 15 weeks. In cats, the uterus could still be identified at 28 days with ultrasound but wall layering was not seen. It is a judgment call in the early stages to decide whether involution is proceeding normally or not. It may be helpful to perform serial examinations to determine if involution is occurring.

Cystic ovaries

The ovary will not be appreciated on radiographs unless it is very large as it could be when a large cyst is present. Cystic ovaries may be associated with pyometra, cystic endometrial hyperplasia, or hydrometra. Radiographically, a fluid opacity is seen near or at the caudal aspect of the kidney. On sonography, cysts have anechoic contents, a distinct thin distal wall, deep acoustic enhancement, variable size, and can be unilateral or bilateral. Smaller cysts can be confused with follicles or corpora lutea. Additionally, inactive cysts cannot be distinguished from those that produce hormones on the basis of ultrasonography.

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Imaging of the small animal male reproductive system

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Abstract

Although radiography is useful in some instances to diagnose enlargement of the prostate, the cause of enlargement is not always obvious. It may also be difficult to determine whether a caudoventral opacity is the bladder or prostate. Ultrasonography can help differentiate between bladder and prostate and can characterize disease conditions within the prostate such as benign prostatic hyperplasia, prostatitis, abscesses, cysts, and neoplasia. Additionally, ultrasonography can be used to evaluate the kidneys, ureters, urethra, and lymph nodes which may be affected. Testicular lesions can also be evaluated with ultrasound. Radiography should be used to check for pulmonary metastasis when neoplasia is suspected.

Keywords: Prostate, testicle, ultrasonography, radiology

Normal male

On radiography, the prostate gland of an intact mature male is usually found cranial to the pelvic floor on the lateral projection. On the ventrodorsal projection, care should be taken to include the pelvic region, looking for the edge of the pubic bone, not the ilial wings to avoid exposing the radiograph too far cranially. The prostate is usually not visible in immature males or males that have been neutered at a young age.

For sonography of the prostate gland, the dog can be placed in either dorsal or lateral recumbency. With the dog in dorsal recumbency, the penis and prepuce can be displaced laterally to allow the probe to be aligned with the long axis of the bladder. With the dog in lateral recumbency, the probe needs to be held at about 45° to the long axis of the body to allow the beam to be oriented along the long axis of the bladder. For immature or neutered males, it may be necessary to tilt the probe so that the beam points dorsally into the pelvis. A high frequency transducer can usually be used because the prostate is superficial in most cases. A lower frequency transducer may be necessary for an enlarged prostate. To image the bladder in the transverse plane, the bladder is imaged as a circle or oval. Then the probe is slid caudally over the bladder trigone and urethra to the center of the prostate. When imaging the prostate in the longitudinal plane, the probe should be swept from side-to-side. The urinary tract and medial iliac lymph nodes should be evaluated also.

The normal prostate in the intact male should have a uniform, somewhat coarse echogenicity, similar to that of the spleen. The gland should be slightly hyperechoic especially in areas with high collagen content. Gas in the colon can create hyperechoic artifacts which should not be confused with disease because of side lobe or slice thickness artifact. The lobes of the prostate should have a similar size. In longitudinal images, the prostate should be round or oval with a smooth symmetrical shape. The urethra and urethralis muscle are seen as a linear hypoechoic area centrally. In transverse images, the two lobes are seen on either side of the central urethral region. The size of the prostate increases with age and size of the dog. In the neutered or immature male, the prostate is small, oval, walnut-sized, and hypoechoic. The bilobed nature of the prostate may not be obvious in transverse images. Tom cats have a diffuse prostate, which is very small and is usually not seen. Prostate problems are unlikely to occur in the cat.

Positive contrast urethrography can be useful to reliably determine the location of the urinary bladder when ultrasonography is unavailable. Margins of the urethra should be smooth. Although a small amount of contrast may enter the prostatic ducts, pockets of accumulated contrast should not be seen within the parenchyma. Conversely, fluid pockets such as cysts and abscesses may not communicate with the prostatic ducts so that a lack of contrast-filled areas cannot rule out these lesions.

It is not necessary to clip the scrotal hair in order to image the testicles. Because the testicles are small, a high frequency transducer is generally used. On a longitudinal image, the testicle is smooth,

homogenous, and elliptical. The rete (mediastinum) testis is hyperechoic and runs longitudinally through the testicle. The testis may be hypoechoic deep to the rete testis in both longitudinal and transverse planes. The epididymis can be followed as a hypoechoic structure running dorsally along the testis with the coarsely hypoechoic head and tail at either end. The vaginal tunics and tunica albuginea present as a combined hyperechoic structure seen peripherally. The pampiniform plexus shows as anechoic tortuous vessels dorsal to the head of the epididymis. Masses in the testicles are common and may or may not be significant.

Abdominal masses

Masses that might be seen in the mid-abdomen include a splenic mass (hemangiosarcoma, hematomas), a pedunculated mass from the liver, an enlarged lymph node, an intestinal mass, and in the male, a retained testicle. Dorsocaudal masses can be important in that they may indicate enlargement of the medial iliac lymph nodes. Enlargement of these nodes can signal inflammation or neoplasia in the prostate gland or bladder. Masses in the ventrocaudal region could be associated with enlargement of the prostate gland. Differentials for enlargement of the prostate gland include benign prostatic hyperplasia, prostatic neoplasia, prostatic abscess, and prostatic/paraprostatic cysts.

Benign prostatic hyperplasia

On radiography, the prostate gland is larger than expected as the volume increases in intercellular and ductal spaces. With benign prostatic hyperplasia (BPH), the prostate can be up to 4x its normal size. If the urinary bladder is full, two fluid opacities will be seen in the caudoventral abdomen. The more cranial opacity (the urinary bladder) will have a pear-shaped appearance and the caudal aspect will blend into the fluid opacity of the more caudal prostate gland. If the urinary bladder is empty, only one opacity may be apparent. Initially, with significant enlargement of the prostate gland, a triangular fat pad is apparent between the bladder cranially, the prostate caudally, and the abdominal wall. This fat pad becomes compressed and no longer visible when greater enlargement occurs. Dorsal displacement and compression of the colon occurs as the prostate enlarges.

On ultrasonography, there is usually symmetrical, hyperechoic enlargement and margins of the prostate appear smooth. Cysts are often seen in the parenchyma. On occasion, the gland can be asymmetric or nodular but there is greater concern for infection or neoplasia when the prostate has a complex appearance or marked asymmetry.

Prostatitis

Prostatitis can occur as a secondary complication of BPH and is hard to differentiate from uncomplicated BPH. Radiographically, the prostate gland is enlarged with an appearance similar to BPH. The sonographic appearance of prostatitis is also similar to that of BPH so that the two conditions cannot be differentiated on the basis of ultrasound alone. On sonography, the prostate gland may be somewhat more echogenic and inhomogeneous with prostatitis than with BPH. Although dystrophic mineralization can occur in chronic cases, mineralization should always be investigated as this can be a sign of neoplasia. Significant enlargement of the medial iliac lymph nodes should also sound an alarm. Some adjacent fluid can be present in acute cases.

Prostatic neoplasia

When an enlarged prostate gland is seen radiographically, care should be taken to look for warning signs of neoplasia. These include asymmetry, mineralization on the vertebrae or pelvic bones or in prostate/urethra, thickening of the adjacent abdominal wall, enlarged medial iliac lymph nodes, or pulmonary metastasis.

On sonography, margins of the prostate gland may appear asymmetric and irregular. Rather than uniform echogenicity, the prostate gland may have mixed echogenicity giving it a complex appearance. Hyperechoic areas with deep acoustic shadowing may indicate mineralization. The medial iliac or hypogastric lymph nodes may be enlarged and irregular with mixed echogenicity indicating metastasis.

Osseous proliferation may be apparent on the ventral aspect of the bodies of the caudal lumbar vertebrae. Changes may extend caudally into the urethra or cranially into the urinary bladder. If the trigone region is involved, obstruction of one or both ureters could result in hydroureter and/or hydronephrosis. Always check both "upstream" and "downstream." Thoracic radiographs should be exposed to evaluate for possible pulmonary metastasis. Aspiration or biopsy can be done to confirm neoplasia. Adenocarcinoma and transitional cell carcinoma are two of the more common neoplasms identified.

Urethrography can be used useful if ultrasonography is unavailable to evaluate the prostate. Large or irregular pockets of contrast that accumulate within the gland suggest neoplasia. Additionally, double contrast cystography and excretory urography can be used to investigate the bladder and kidneys respectively.

Neoplasia is a significant problem in neutered males. In males that have been neutered young, the prostate gland should be a small hypoechoic oval structure with smooth margins. Any evidence of mineralization or irregularity should be investigated. It the male is older and has been recently neutered, hyperechoic areas may represent resolving BPH. Acoustic shadowing suggests mineralization, which should be investigated. In either case, involvement of the urinary bladder or urethra suggests neoplasia.

Prostatic abscess

On radiography, smaller abscesses may not be apparent if they do not distort the margins of the prostate gland. Larger abscesses can cause the prostate to be enlarged and misshapen, suggesting the presence of neoplasia. On ultrasonography, an abscess is anechoic-to-hypoechoic. Abscessed areas may appear to contain cellular debris, which may swirl within the gland during real-time sonography. Hyperechoic areas in the gland may be caused by fibrosis, gas, or mineralization.

Paraprostatic/prostatic cyst

Cysts within the prostate are common and are often present in benign prostatic hyperplasia. Larger cysts are anechoic and may exhibit deep acoustic enhancement. Smaller cysts are more likely to be hypoechoic. Cysts that extend from the prostate gland may cause the impression of three bladders on radiography. Some cysts may appear cranial to bladder. Sonography can be useful to confirm the diagnosis and identify the real bladder. Most extra-parenchymal cysts can be traced back to the prostate gland and are prostatic cysts. Rarely, a paraprostatic cyst arises from a persistent Müllerian duct (uterus masculinus). A remnant of the Müllerian ducts or extension from prostatic lobe may be attached to the prostate by thin stalk or broad fibrous bands. Cystography can be performed to identify the bladder if sonography is not available.

Retained testicle

Retained testicles are often found cranial to the urinary bladder but may also be located at the inguinal ring or at the caudal aspect of the kidney. Smaller retained testicles will not be visible on radiography. Retained testicles may become neoplastic and quite large and may be visible radiographically. On sonography, some retained testicles appear similar to testicles found in the scrotal sac. Others are small and poorly formed. If present, the hyperechoic rete testis can help to identify the testicle. The tubular gubernaculum testis is sometimes seen extending from the caudal aspect of the testicle to the inguinal ring.

Testicular nodules and masses

Neoplasms in the testicle may be hypoechoic, hyperechoic, or complex. Although most nodules are benign, other nodules could represent a malignant neoplasm. The sonographic appearance of solid masses is non-specific and aspiration or biopsy is necessary to determine the nature of lesions. If the urethra cannot be readily identified, a catheter can be placed to determine its location allowing it to be avoided during the procedure. It is also important to realize that disease can be isoechoic. If a lesion doesn't alter the reflective properties of the tissue, no echo will occur.

Testicular neoplasms include Leydig cell tumors, interstitial cell tumors, Sertoli cell tumors, and seminomas. Leydig cell tumors and interstitial cell tumors are usually benign while Sertoli cell tumors and seminomas have a greater potential for malignancy. Interstitial cell tumors are composed of small nodules that may become confluent. They are poorly encapsulated, may be associated with hormone changes, and may be bilateral. Sertoli cell tumors cause enlargement of the affected testicle while the other testicle usually becomes atrophied. This type of tumor may be hypoechoic and may be associated with feminizing syndrome and bone marrow suppression. The seminoma may be large, solitary, and unilateral with internal necrosis and hemorrhage. No hormone change is associated with this tumor. Tumors that occur in retained testicles are more likely to be malignant than those that are found in a testicle located in the scrotum.

Testicular cysts

Cysts are classically anechoic with deep acoustic enhancement and a well-defined far wall. Smaller cysts will appear hypoechoic rather than anechoic. Complicated cysts are hypoechoic due to infection or hemorrhage.

Orchitis

Inflammation or infection can cause the testicle to be increased or decreased in echogenicity. Initially, the testicle may be enlarged but the testicle may become atrophied and small if chronic changes occur. Fluid might be present in the scrotal sac or there may be scrotal thickening. Abscesses may be present in some cases. Prostatitis or urinary tract infection might also be present.

Testicular atrophy

The testicle may be smaller than normal due to aging change or as the result of disease. It may be hyperechoic-to-isoechoic on sonography.

Testicular torsion

The testicle may rotate causing vascular obstruction. With torsion, the testicle becomes enlarged with a swollen epididymis. On sonography, a decreased Doppler signal confirms compromise of the vasculature. Torsion is particularly likely to occur in retained testicles that have become neoplastic.

Preputial lesions

Variable lesions include cysts, edema, and neoplasia. Changes in opacity or echogenicity can be seen with these. Ultrasonography can be useful to further characterize the lesion and to help in a suitable area for aspiration.

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Windows of opportunity: a new look at canine socialization

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Veterinarians and trainers often recommend "socialization" to owners of young puppies as part of standard education and care. Most recommendations are focused on exposing the puppies to a variety of stimuli including children, novel objects and body handling. It is important to recognize that socialization outcomes are affected by more than just these experiences. Long term socialization and adult behavior patterns can be affected by genetic influences as well as by specific experiences during multiple developmental stages including the prenatal, neonatal and early socialization periods.

Genetic influences

Much information can be learned from the results of experiments conducted by the Russian geneticist Belyaev on silver foxes. Following selective breeding for behavioral traits associated with tameness and domestication, the researchers observed changes in the physical and physiological appearance as well as in the behavioral characteristics of the offspring.

Behavioral changes included increases in solicitation of contact and social interaction from humans, increases in licking behavior directed towards human hands and faces, and increases in tail wagging and other care soliciting behaviors. The socialization period of the selected offspring was extended from the normal 40-45 day limit until approximately 60-65 days.[1] Physical/physiological changes included a shift to twice annual reproductive cycling, drooped ears, erect tail carriage as well as changes in coat texture and color.

Additional studies involving cross-fostering and cross-implantation showed that the aggression level of the offspring was primarily determined by the genetic makeup of the individual rather than by the gestational or postnatal environments and social interactions. An overview of the information gathered from several decades of research on silver foxes was recently published.[2]

Genetic information has also been gathered from a line of pointers affected by inbreeding and selection pressure for "nervous" traits. These dogs show excessive timidity, exaggerated startle responses, a reduction in exploratory behaviors and increased "freeze" responses in response to exposure to humans or novel stimuli.[3] Enlarged adrenal glands and other physiological changes have been described in the affected dogs,[4] suggesting a similar connection between behavior, genetics, and physiology as in the silver fox. Affected animals have been used in research as a model for human psychopathology. A recent review article covering additional information about the connections between genetics and behavior is available.[5]

A study conducted in 1985 collected opinions regarding behavioral differences between 56 breeds of dogs. While this information is based on individual opinions rather than direct behavioral observations, comparisons between various breeds suggest that some traits such as trainability or protectiveness are consistently higher or lower in some than in others.[6] This suggests that breed specific genetic traits can have a significant impact on adult behavior patterns and shouldn't be overlooked when considering the influences on social behavior in dogs.

Prenatal effects

The hypothalamic - pituitary - adrenal (HPA) axis is a major part of the neuroendocrine system. Corticotropin releasing hormone (CRH) is released from the hypothalamus in response to stress. This triggers the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which leads to the release of cortisol from the adrenal cortex. Cortisol is responsible for mediating the body's alarm response to stress. Circulating levels of cortisol exhibit a negative feedback influence on the release of both CRH and ACTH. Persistently elevated levels of cortisol are commonly associated with chronic stress or anxiety; abnormal responses of the HPA axis have been associated with a variety of pathological conditions including post traumatic stress disorder, generalized anxiety and irritable bowel disorder.

The effects of prenatal stress on development of the fetal HPA axis have been explored in multiple laboratory species including rats and non-human primates. While the same research has not been completed on dogs, these studies suggest that prenatal stress during specific developmental periods can alter the formation and development of the fetal HPA axis and predispose the offspring to reactivity, hyperactivity and disorders related to attention or focus.[7] These effects have been shown to occur independently of genetic selection pressure and are present in cross fostered offspring as well as in naturally reared animals, indicating that the effects are due to prenatal rather than postnatal factors.[8]

Neonatal period

This developmental period in domestic canines lasts from birth until the eyes and ears of the puppy open at approximately two weeks of age. During this time, the physical and neurologic state of the puppy is immature and incompletely developed. Ongoing neurologic development and maturation can be affected by tactile, thermal and positional experiences during the first few weeks of life.

Brief periods of handling consisting of exposure to mild thermal stress, vestibular stimulation on a tilting board, exposure to flashing lights and auditory stimulation from birth to five weeks of age were associated with several observations. The pups that were exposed to handling and these environmental changes were found to be more confident, engaged in more exploratory behavior, displayed earlier maturation of the nervous system, gained weight more rapidly and showed decreased emotionality, among other findings.[9]

Similarly, the "Super Dog" or "BioSensor" program of the US military was developed to improve the performance of dogs used for military purposes. This program, also described as Early Neurologic Stimulation, consists of five exercises performed in succession: tactile stimulation between the toes, holding the pup with the head erect, holding the pup with the head pointed down, holding the pup in the supine position, and exposing the pup to thermal stimulation (pup is placed on a damp towel that was cooled in a refrigerator for at least five minutes). The exercises were recommended to be performed once daily on days 3-16 and for no longer than 3-5 seconds each to avoid possible detrimental effects associated with prolonged or chronic stress. Puppies exposed to this program were reported to have improved cardiovascular performance (measured by heart rate), stronger heart beats, stronger adrenal glands, improved tolerance to stress, and greater resistance to disease.[10]

Primary socialization period

During this period from three weeks until approximately 12 to 16 weeks, puppies begin to seek out social interactions other than with their mother, setting the stage for the development of species-specific social behaviors. Puppies also undergo additional maturation of motor and neurologic patterns as they form primary associations with their physical and social environments.

In general, the period from three to five weeks is characterized by rapid recovery from fearful incidents and the puppies begin to actively approach unfamiliar individuals. Nutritional and social weaning occurs between six and fourteen weeks as the mother becomes less tolerant of the puppies and encourages independence and self-sufficiency. Anecdotal reports suggest that puppies go through a "fear period" sometime between 12 and 14 weeks; this may have evolutionary advantages for the puppies but has the potential to interfere with positive socialization efforts.

Observations of puppies who were isolated from humans, other dogs, or who were raised in a kennel environment until 14 weeks showed behavior patterns of anxiety, active avoidance and generalized fear.[11] In addition, if a puppy is socialized appropriately, but then is isolated from three months onward, the puppy is less capable of learning new behaviors as well as decreased retention of previously learned behaviors.

Active socialization should include a wide variety of experiences such as exposure to new locations, different walking surfaces, social interactions with people of all ages, genders and skin tones, social interactions with dogs of various breeds and ages, as well as introductions to novel objects and body handling exercises. It is important that owners pay attention to the quantity as well as the quality of

the experiences to avoid overwhelming or creating conditioned fear responses in shy or more vulnerable puppies.

Ongoing learning

As puppies make the transition to new homes around the age of eight weeks, active socialization is ongoing and new pet owners need to be educated about their role in this process. Puppies actively learn new skills such as crate training, basic commands, house training, bite inhibition, and social independence. It is important that new pet owners are supported through this process by knowledgeable and timely information from veterinary staff members, breeders and trainers.

Training methods should focus on positive reinforcement methods while avoiding or minimizing the use of punishment. Training should provide a foundation of basic obedience such as learning to "sit", "down" and "stay" on command, and should also include other exercises to develop impulse control and teach household manners with guests and in social situations. Adolescent dogs are naturally more boisterous, impulsive and independent which means that these training exercises should be started in puppyhood and continued through the period of social maturity from one to three years of age.

Because adult behavior can be impacted during each of these "windows of opportunity" it is important that veterinary professionals and animal care providers understand these influences and can provide appropriate recommendations to breeders, trainers and pet owners during each of these periods.

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Reproductive disorders of the spayed bitch

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Abstract

Ovariectomized bitches will often present to veterinarians for signs of vaginal discharge, recurrent or returning signs of estrus, or for vaginal masses. These may be due to presence of remnant ovarian tissue or other sources of estrogen exposure, vaginitis, vaginal foreign bodies, anatomic defects of the vaginal canal, or from other metabolic conditions. This paper reviews causes of vulvar discharge, ovarian remnant syndrome, stump pyometra or granuloma, and the different types of vaginal masses that may be seen in the ovariectomized bitch. It provides insight into the clinical signs, diagnostic workup and therapies that can be utilized to diagnose and treat these disorders.

Keywords: Ovarian remnant syndrome, vulvar discharge, vaginitis, vaginal mass, spayed

Introduction

Veterinarians are frequently presented spayed bitches with reproductive disease. The most common complaints are vulvar discharge, recurrent or returning signs of estrus, or vaginal masses. Diagnosis of these disorders may be straightforward in some cases, but they are often complicated and confusing. A step-wise approach to their diagnosis begins with a good history paying careful attention paid to nutrition (type of food being fed) along with any supplements or medications the bitch may be administered purposefully or that she may be exposed to via the owner's personal use. A complete physical examination, followed by a minimum database of bloodwork and a urinalysis should follow. Ancillary diagnostics may include endocrinology, diagnostic imaging consisting of ultrasonography, radiography with or without contrast medium, magnetic resonance imaging, endoscopy and possibly laparoscopy or laparotomy depending on the situation. Each complaint will be reviewed separately although there is some overlap amongst them. A list of potentially estrogenic foods has been included in Table 1.

Vulvar discharge

A common presenting complaint of spayed bitches is the presence of vulvar discharge with or without perivulvar dermatitis. This discharge may be mucoid, mucopurulent, purulent or bloody and it may be chronic or intermittent. Causes of vulvar discharge in the bitch typically originate somewhere in the genitourinary system and can be due to infection (bacterial or viral; chronic or acute), chemical irritation from urine or other instilled substances, foreign bodies, neoplasia (vaginal, cervical or uterine), stump pyometra or granuloma, ovarian remnant syndrome (with or without ovarian cysts or neoplasia), congenital anatomic defects or trauma.[1-13] There are two reports of vaginal discharge associated with non-reproductive tract neoplasia.[1,2,14] It has also been reported that endocrine disease such as diabetes mellitus or chronic pyoderma may also have vaginitis as a presenting sign.[1-3] Vascular anomalies (hamartoma and vascular ectasia) have been described in two case reports presenting with bloody vulvar discharge.[15,16] Coagulopathies may also present with vulvar bleeding.[2,3]

Clinical signs may include excessive licking or pruritus, stranguria, hematuria, dysuria, pollakiuria, incontinence, polyuria, and polydipsia.[1-3,6,13,17] Discharge may accumulate on the perineum or haircoat.[1,17] Vaginal discharge has been reported to be mucoid in about a third of the cases (33%), mucopurulent in 20% of the cases, and purulent in just over a quarter of the cases (27%).[1,2,13] Discharge is typically blood tinged in cases of ovarian remnant syndrome or coagulopathy.[2]

The initial diagnostic plan following physical examination should start with a diagnostic vaginal cytology to characterize the discharge.[1-4,13,17] This cytology should be obtained using some type of guarded technique to avoid vulvar, clitoral or vestibular contamination.[17] Sedation may be necessary in some spayed bitches to obtain vaginal samples. Particular attention should be paid to the types of vaginal cell present to determine if there is any estrogenic influence present (superficial cells should not be

present in any spayed bitch). Further attention should be paid to the types and number of bacteria as well as the presence of neutrophils (degenerate or non-degenerate) and whether there is phagocytosis of bacteria present.[1] Normal, spayed bitches will have predominantly basal and parabasal cells with low to moderate amounts of mucus, low numbers of neutrophils and low numbers of bacteria (rods and cocci) with none intracellularly.[17] Cultures should be obtained with a long, guarded culture swab.[2] Cultures should be submitted for aerobes, Mycoplasma and Ureaplasma. A urinalysis (sterilely obtained, preferably by cystocentesis to avoid vaginal and vestibular contamination) should be performed to rule out the urinary tract as the source of infection in any bitch with signs of vaginitis.[2,3,17] Urine culture may also be necessary to rule out a subclinical cystitis.[2] When lymphocytes predominate, an immunologic etiology may be present.[1,4] Eosinophilia may also be present in these bitches.[1] A complete blood count and serum chemistry should be performed to identify inflammation, anemia, or signs of endocrine disorder and a prothrombin time, partial thromboplastin time and buccal mucosal bleeding time should be performed to rule out coagulopathy.[3]

The bacterial flora of the vagina has been well documented and it is not abnormal to culture a variety of bacteria from the bitch's vaginal canal (Table 2).[1-4,18-25] It can be difficult at times to determine if a pathologic bacterial population is present versus normal vaginal flora, so the use of cytology in conjunction with culture to provide clear evidence of inflammation (phagocytosis of bacteria) is imperative.[1-3,13,17] Bacterial growth is considered more likely to be significant when there is growth of a single, or possibly two organisms, with moderate to heavy growth.[2] Infectious vaginitis has been shown to result in isolation of heavy growth of a single organism in 27-40% of cases.[13,19,26] Mycoplasmas have been isolated from 88% and Ureaplasmas from 50% of normal bitches; while more bitches with infertility present with Ureaplasmas (75%), compared to those with no signs of reproductive disorders (40%).[24] Brucellosis may cause vaginal discharge.[2,3]

Viral infection with herpesvirus may cause vesicular vaginitis which can be diagnosed on endoscopic examination.[2-4,17,27] These bitches may present with excessive licking, dysuria, or pollakiuria, with a serosanguinous or sanguinous vulvar discharge.[27] Visual inspection of the mucosal surface of the vagina and vestibule, as well as the mucocutaneous junctions of the vulvar lips, will reveal either vesicular lesions or round, ulcerated lesions that have already ruptured.[2-4,27] Lymphofollicular hyperplasia may be seen in the vagina or vestibule and may be either pathologic or a normal finding.[4] Viral inclusion bodies may be seen on cytologic examination.[4] Treatment with topical antiseptics have been advocated, but most of these cases will resolve on their own in a few weeks if left untreated.[2,3,27] Herpesvirus may recrudesce and cause similar signs in the future particularly during times of physical or physiologic stress.

Vaginoscopy is very helpful to assess the appearance of the mucosa as well as to obtain biopsy samples where appropriate.[1-7,10,13] The use of an otoscope speculum allows visualization of the caudal vagina and vestibule only. A pediatric proctoscope or endoscope (cystoscope or ureteroscope) is needed to evaluate the vaginal canal proximal to the vestibule-vaginal junction. Digital examination of the vagina is also necessary, as strictures and septa may easily be missed during vaginoscopic examination as the endoscope is small and easily slips past any narrowings.[1,2,5-7] Sedation may be necessary for these examinations in spayed bitches.

Anatomic defects of the vulva, vestibule and vagina can cause vaginitis.[1-11,13] Vestibulovaginal strictures or stenosis, vaginal septa, hymenal remnants and juvenile or hooded vulvas can cause pooling of mucus or urine in the vaginal canal resulting in infection, inflammation or chemical irritation, incontinence or urinary tract infection.[1-10] Endoscopy and/or radiography with contrast medium are necessary for diagnosis.[1-3] Adult onset clitoral enlargement is likely due to exposure to either endogenous (adrenal or ovarian remnant origin) or exogenous androgens (either via direct administration, nutritional supplement or exposure to an owner's medicated cream or patch).[3,4]

Treatment of vaginitis should include appropriate antibiotic therapy, based on culture and sensitivity, only if clear evidence of infection is present.[1,3,17] Many cases of vaginitis will resolve without any treatment within a few months of presentation.[1-3,13] If phagocytosis of bacteria is not evident, the use of antibiotics may disturb the normal vaginal flora and worsen the condition.[28] Douching is an effective treatment for non-infectious vaginitis.[4] This author uses a sterile physiologic saline solution for large

volume douching and then follows with a 1% acetic acid rinse in the last one or two rinses. Initially douching may need to be performed more frequently to clear the tenacious mucoid secretions from the walls, but then typically can be performed with less frequency as the discharge resolves. The author has also found that the use of probiotics, particularly those with *Enterococcus faecieum*, can help re-establish normal vaginal flora. In the author's experience, in cases of chronic vaginitis due to vaginal or vulvar anatomic defects, douching may be needed intermittently for the patient's entire life and probiotics may be needed daily. Resolution of signs may take months to years, or may remain for the remainder of the pet's life and need to be treated intermittently.[1] In some cases, administration of glucocorticoids may help resolve chronic, non-infectious vaginitis.[1] Bitches with incontinence may respond to treatment with phenylpropanolamine, estriol or diethylstilbestrol.[1-3]

Surgical treatment of anatomic defects may result in resolution of signs.[1-3,6-9,11,13,29] Bitches that are over-conditioned with a hooded or juvenile vulva, should be encouraged to lose weight prior to surgery and if signs do not resolve with weight loss alone, episioplasty can be performed.[1-3,7,8,11] Surgical removal of hymenal remnants may be beneficial.[1-3,11] Laser ablation of strictures or septa has been described.[10] Surgical removal of strictures via vaginectomy or vaginal anastamosis and resection have been described but the owners should be warned that healing may result in worsening of the stricture as well, so may not provide a cure.[1,5,6,9,11,29] Clitorectomy may also be beneficial in cases where a persistent clitoritis is confirmed.[1]

Ovarian remnant syndrome

Ovarian remnant syndrome is a condition where functional ovarian tissue remains in a previously gonadectomized female.[30-32] Bitches may have signs of estrus return within weeks to months of their spay surgery or it may be many years before signs present.[30-33] Bitches with ovarian neoplasia in the remnant tissue have a tendency to take longer to display signs of estrus compared to bitches with normal ovarian architecture.[31,32] It has been reported to have an incidence of up to 17-43% of all complications of ovariohysterectomy.[32,34] Most reports indicate that cats are overrepresented compared to dogs[30,33,34] but one report showed a higher number of canine cases compared to feline.[32] This may be due to the caseload of the clinic or the types of cases referred to this particular institution during the time frame studied.

Etiology

Failure to identify the ovary in its entirety may occur due to the fatty bursa surrounding the ovary in the bitch, particularly in anestrous bitches where the ovary can be very small.[30,32] Improper placement of clamps, ligatures or Ligaclips® during surgery may result in a portion or the entire ovary to be left behind.[30-32] Inadequate exposure of the ovary may occur due to failure to break down the suspensory ligament of the ovary or an abdominal incision that is too small or located too posteriorly.[30-32] Accessory ovarian tissue has been reported in queens, women and cows, but not in bitches.[30-32] There is no known breed or age predilection.[30,32,33] In cats, it has been shown that a piece of ovarian tissue may re-establish a blood supply and become functional again if the ovarian tissue is loosely attached to the intestinal mesentery.[35] It took only six months to re-establish follicular activity in these animals.[35] So it is possible that if a portion of an ovary is dropped into the abdomen of a dog it may re-establish a blood supply and become a functional ovarian remnant.

There are five case reports of ovarian tumors in previously spayed bitches with ovarian remnant syndrome: four report granulosa cell tumors (GCT)[14,36-38] and the other was combination of a luteoma and Sertoli cell tumor.[39] There is typically a long period of time (2.5-10 years range but 6-9 is average) from ovariectomy to initiation of signs of estrogenization in bitches that develop GCT in their ovarian remnant tissue.[14,36-38] Similarly, the luteoma/Sertoli cell tumor did not show clinical signs for ten years.[39] Persistently elevated levels of gonadotropins may contribute to tumorigenesis.[38] Granulosa cell tumors can produce both estrogens and progesterone.[14,36-38] The estrogen secreted by the tumor may result in the typical signs of estrogen expression (vulvar swelling and discharge, attraction of males, etc) and this may be intermittent or persistent.[14,36-38] If progesterone is also produced by the tumor, cystic endometrial hyperplasia may develop and if there is uterine body left, may eventually develop into pyometra.[36-38] This

elevation in progesterone may be due to partial luteinization of granulosa cells due to the persistent estrogen secretion by the tumor.[38] In humans, GCTs express gonadotropin receptors and respond to gonadotropins.[38]

Clinical signs

Clinical signs will vary depending on how much of the reproductive tract was left behind at the time of ovariohysterectomy.[30] Vulvar edema, flagging and receptive behavior, and attraction of male dogs will be noted in the majority of cases, while bloody vulvar discharge will be seen only in those which have uterine tissue remaining.[30-32] Some bitches will display mammary development and lactation associated with pseudocyesis.[31,32] Cyclic behavior will be typical of an intact animal (four to 14 months between cyclic behaviors).[30,31] Some studies showed the right ovary is more commonly left behind compared to the left ovary and this may be due to its more cranial location and thus greater difficulty in exteriorizing it fully.[30,32,34] Other studies did not have this finding which may be due to the population of animals examined.[33] It has been reported that ovarian remnants are not more common in deep-chested or obese individuals and this may be due to additional efforts made by the surgeon to obtain good exposure in these patients.[30,32,33] It has also been reported that ovarian remnants are not more common in bitches with pyometra or that are spayed at the time of a cesarean section and this may be due to the stretch on the suspensory ligaments with these conditions allowing better ovarian exposure.[30,32,33] Indeed, most remnants occur after elective ovariohysterectomy, often in pre- or peri-pubertal individuals, probably due to small ovarian size and the desire to use as small an incision as possible, thus making exposure more difficult and identification of the ovary proper more challenging.

Endocrinology of intact versus neutered animals

Estradiol concentrations are too variable to use to classify a bitch as intact or gonadectomized.[40] Estradiol is produced via aromatization of androstenedione and testosterone in peripheral tissues. Estradiol is not produced by the adrenal glands.[40] Progesterone concentrations rise more significantly in intact anestrus bitches following cosyntropin injection, than in gonadectomized bitches (Table 3).[40] Progesterone can be produced by the adrenal glands so an ovariectomized animal can have a low level of progesterone present.[41] Because there may or may not be a significant response to gonadotropin releasing hormone (GnRH) to stimulate release of progesterone or testosterone, these hormones are not recommended for testing purposes.[42] Gonadotropin releasing hormone causes an increase in estradiol concentrations in intact bitches but not in ovariectomized bitches.[42] Concentrations of luteinizing hormone (LH) and follicle stimulating hormone (FSH) are significantly higher in ovariectomized vs. intact bitches due to lack of negative feedback on the hypothalamus and pituitary via the ovaries.[42-44] In one study, concentrations of LH were typically >8 ng/ml in ovariectomized bitches.[44] In the same study, FSH concentrations in intact (mean 98±49 ng/ml) and ovariectomized (mean 1219±763 ng/ml) were significantly different.[44]

Gonadotropin releasing hormone stimulation results in a significant increase in LH in ovariectomized and anestrus bitches; whereas GnRH stimulation does not cause a significant increase in FSH in ovariectomized bitches while it does in anestrous bitches.[42,43] This is most likely due to alterations in intracellular storage of FSH compared to LH.[43] Gonadotropin releasing hormone stimulation testing of FSH may provide a diagnostic tool for distinguishing ovariectomized from intact bitches by the lack of response in ovariectomized bitches. While FSH may be valuable for determination of spay/neuter status, few laboratories have assays available commercially, so it is very difficult to use on a routine basis. It is extremely important that if FSH testing will be used that the laboratory's assay be validated for canine serum.

Diagnosis

Vaginal cytology will confirm the presence of estrogen during the follicular phase of the cycle or if the bitch has steroidogenically active follicular cysts or neoplasia of the ovarian remnant.[30,32] The presence of nucleated or anucleated superficial cells is consistent with estrogenic stimulation. Exposure

to or administration of exogenous estrogens, either intentionally (diethylstilbestrol or estriol for incontinence) or unintentionally (exposure to the owner's estrogen supplements, creams or patches or supplementation of estrogen containing foodstuffs) may result in signs of estrus without a remnant being present and this must be differentiated during diagnostic testing.[30,32]

Other causes of bloody vulvar discharge need to be ruled out including supernumerary or accessory ovaries, neoplasia, vaginal foreign bodies, vaginitis, stump pyometra, trauma, and coagulopathy.[30,31]

Ultrasound examination. Ultrasonography may be used before or after stimulation tests to detect ovarian tissue, cysts, or neoplastic ovarian tissue.[31,32] Very small remnants may be missed with ultrasonography and the lack of finding tissue compatible with ovarian tissue, does not rule out the diagnosis. Suture granulomas may be confused with an ovarian remnant. A diagnosis may be made by ultrasonography only a portion of the time (50-75%).[32,45]

Baseline endocrine testing. Estradiol concentrations can be determined when the bitch shows signs of bloody discharge, vulvar edema, or receptive behavior.[30-32] It should be remembered that estrogen concentrations are highest during the follicular phase of the cycle and that once the LH surge happens, estrogen concentrations drop quickly. If samples are not obtained during the follicular phase of the cycle, they may not be diagnostic.[30] There is considerable overlap of normal ranges between intact and ovariectomized animals, so in some cases, estradiol concentrations may be low enough that a diagnosis cannot be confirmed. Keep in mind that exogenous estrogen exposure may result in elevated estradiol concentrations and may mislead the veterinarian to believe there is a remnant present when there is not.[30] For this reason, stimulation testing is usually recommend over a single baseline estradiol concentration. Adrenal production of estrogens is also possible and may be seen with adrenal neoplasia or adrenal disease. Estradiol concentrations fluctuate throughout the day, so a single sample may be misleading if it is at a nadir.[30] Basal estradiol concentrations are detectable in bitches with remnant ovarian tissue but not in ovariectomized bitches.[45] Basal estradiol levels are higher in bitches with remnant tissue than in intact anestrus bitches.[45]

Progesterone concentrations will be elevated (>2 ng/ml or 6.28 nmol/L) after ovulation occurs.[30-32] Depending on the size of the remnant, concentrations of progesterone may be much higher or may remain in this low range during the luteal phase of the cycle, which may last from 45 to75 days after ovulation.[30,45] Low levels of progestagens can come from the adrenals but levels over 2 ng/ml can only be produced by functional ovarian tissue or by exogenous progestagen exposure.

Both estrogen and progesterone may be affected by lipemia and hemolysis, so samples should be taken following a six to eight hour fast and should be drawn atraumatically, placed in plain red top tubes, centrifuged within 30 minutes of sampling and the serum removed from the red blood cells and then either refrigerated or frozen depending on length of time until testing (<24 hours or>24 hours, respectively).

Luteinizing hormone is secreted episodically at lower concentrations in intact bitches throughout the estrous cycle and then in a very high pulse at the time of LH surge during late proestrus.[44,46] Secretion of LH increases after gonadectomy as a result of diminished negative feedback, but it still remains pulsatile in its release pattern.[46] Basal LH concentrations are significantly higher in ovariectomized bitches than in anestrous bitches.[42,46,47] A single positive LH test provides a positive predictive value that a bitch is ovariectomized only 22% of the time.[47] It may take up to four serial tests to accurately determine spay vs. intact status of a given individual.[47] If a test result is high, repeat sampling another two to three times at two week intervals should help to determine if it is consistently high (ovariectomized) or intermittent (intact).[44] A single negative test following any positive tests is consistent with an intact animal.[47] Serial testing can also be done hourly as episodic pulses of LH are known to occur frequently but do not last more than about an hour, except for the pre-ovulatory LH surge which may last up to 36 hours.[47] Secretion of LH follows a similar pattern in bitches ovariectomized prior to puberty.[47]

Basal LH concentrations are higher in bitches with remnant tissue than in intact bitches in anestrus, but lower than ovariectomized bitches.[45] Basal LH concentrations are higher in bitches with an interval from ovariectomy to appearance of the first estrous cycle after surgery of more than three years, compared to those who display signs in under three years.[45] The higher LH concentrations in bitches with remnant tissue may be due to lower sensitivity to negative feedback hormones as a result of disruption of the blood supply to the remnant by the initial surgical procedure or by partial removal of the ovaries.[45] The diminished blood supply may reduce or ablate ovarian activity for long periods of time, possibly accounting for the delay in resumption of signs in many individuals.[45] The chronic secretion of GnRH because of lack of negative feedback by inhibin from the ovaries, may in turn result in hypertrophy and/or hyperplasia of pituitary cells. These gonadotrophs cells may lose some of their normal characteristics of sensitivity to negative feedback, such that when the remnant tissue becomes active again, LH may not be suppressed to the extent it is in a normal intact bitch, thus resulting in higher basal LH concentrations compared to intact bitches.

Bitches that develop GCT in their ovarian remnant have high circulating estradiol and/or progesterone concentrations.[45] These bitches tend to take longer to begin to show clinical signs of ovarian remnants.[32,45] Basal LH in bitches with ovarian remnants is higher than in anestrous bitches.[38] This high basal LH may be due to loss of sensitivity to the negative feedback from estradiol resulting from partial ovarian removal or partial to complete disruption of the blood supply to the ovaries at the time of ovariectomy or ovariohysterectomy.[38] The resultant lack of ovarian activity for long periods of time results in hypersecretion of gonadotrophs and hypertrophy/hyperplasia of pituitary gonadotrophs cells.[38] As these cells transform, they may lose their sensitivity to negative feedback to the ovarian hormones that are beginning to be secreted from the remnant tissue as its blood supply is gradually re-established.[38] Thus the ovarian hormones cannot suppress LH secretion to the levels typically seen in anestrous bitches.[38] So, in bitches with remnant GCT tissue, estradiol and LH concentrations will be higher than those present in anestrus bitches.[38] It has been hypothesized that another possible reason for increased LH concentrations in bitches with GCT is that there is a positive feedback of estradiol 17-β on LH release, as seen in late proestrus in intact bitches.[38] This hypothesis may be less likely since in the bitch it has been demonstrated that estradiol 17-β exerts negative feedback until its secretion stops (at the end of proestrus) and this is when LH increases.[38,46]

Anti-Müllerian hormone (AMH), also known as Müllerian inhibitory substance, is a protein hormone, produced by the granulosa cells of primary, secondary and early antral follicles.[48] The ovaries are believed to be the sole source of AMH in the bitch.[48] When used in bitches over six months of age, the sensitivity of this assay was 93.9% and the specificity was 93.8%.[48] The advantages of AMH over other types of tests are that it requires only a single basal sample and is not subject to the stage of the estrous cycle.[48] The ability of this test to diagnose ovarian remnant syndrome successfully has not yet been extensively studied, but holds some promise and research is presently ongoing.

Stimulation testing. Administration of GnRH (2 μg/kg) or human chorionic gonadotropin (hCG, 44 IU/kg) will cause luteinization if there is functional mature follicular tissue present.[30-32] If the medication is given too early in proestrus, luteinization may not occur, so it is important to assess vaginal cytology first and to wait until at least 70% of the superficial cells are anucleated before this type of stimulation testing. Progesterone concentrations should be measured two to three weeks after stimulation testing.[30,31] Elevated progesterone at this time indicates functional ovarian tissue. Not all remnants will respond to stimulation testing, so a negative result does not rule out a remnant.

Human chorionic gonadotropin (1500 IU IV) or GnRH stimulation testing (10 μg/kg IV) can be used to determine if an animal has functional ovarian tissue.[37,38,42] Baseline samples and samples taken at 10, 60 and 120 minutes after injection are recommended.[42] Ovariectomized animals have no change in estradiol or progesterone concentrations following stimulation while intact animals should have a two- to four-fold increase, depending on stage of the cycle.[37,42,46] Stimulation testing with GnRH will result in increased LH concentrations at ten and 60 minutes after administration in both intact and remnant bitches, but not in ovariectomized bitches;[42,45] however, there is overlap between ovariectomized and anestrous

bitches though making this an unreliable test.[42] The increase in progesterone and estradiol is lower in remnant than intact bitches.[45] This lowered response again may be due to changes in the gonadotrophs due to altered function from the negative feedback being decreased from altered blood supply to the remnant tissue.[45] It is possible that higher LH concentrations may be found if the animal is tested after 60 minutes, since the response could simply be delayed.[45] Stimulation with GnRH results in an increase in estradiol concentrations at 60 minutes after injection.[42,45] The increase in estradiol after stimulation is higher in anestrus bitches than those with remnant ovarian tissue.[45] Some bitches with remnant tissue may have a lower estradiol concentration 60 minutes after GnRH stimulation.[45] This may be due to cystic changes in some remnant tissue, changes in vascularization of the remnant or be a response to the follicular phase of follicle development in the remnant, since testing is usually performed after the bitch begins showing clinical signs indicating activity in the remnant tissue.[45] Gonadotropin releasing hormone stimulation of bitches with GCT affecting the ovarian remnant results in a significant increase in LH at both10 and 60 minutes after injection and of estradiol at 60 minutes after injection.[38] In intact bitches with GCT, GnRH stimulation results in no significant elevation of LH or estradiol compared to anestrous or ovariectomized bitches.[38]

Treatment

Once a remnant is confirmed, exploratory laparatomy can be performed to remove the remnant tissue.[30-32,34] Surgery is best performed during diestrus because luteal tissue is more prominent in fat than is follicular tissue, but surgery can also be performed during estrus.[30,31] If the remnant is not grossly visible, ovarian pedicle granulomas should be removed and submitted for histopathology.[34] Increased vascularity of the pedicle tissue may indicate remnant tissues.[30,31,34] Any tissue left along the broad ligament should be removed and submitted as well. The abdomen should be explored carefully from the caudal pole of the kidney to the uterine stump on both sides and then the rest of the abdomen should be explored for the presence of ectopic ovarian tissue, since a dropped ovarian fragment may revascularize anyplace in the abdomen.[30,34] Historically, in some countries, a piece of ovarian tissue was allografted into the gastric mucosa near the portal vein drainage area to act as a source of gonadal steroids after ovariohysterectomy to prevent the complications of alopecia and coat changes as well as incontinence issues;[49] thus, it is easy to see how a dropped ovary may revascularize. Any remnants of the uterus or oviducts should also be removed during the exploratory whenever possible.[30-32,34]

Other options for treatment include estrus suppression using mibolerone, megesterol acetate, medroxyprogesterone acetate or Suprelorin® (deslorelin) implants.[30] If uterine tissue remains and megesterol acetate is used to suppress estrus, cystic endometrial hyperplasia may develop and result in pyometra or stump pyometra. Long term use of progestagens may also result in adrenocortical suppression and insulin resistance.[30]

Stump pyometra or granuloma

Stump pyometra occurs as a result of incomplete ovariohysterectomy.[50-53] Pyometra occurs as a result of the priming of estrogen receptors followed by progesterone production. Estrogens and progesterone may be either endogenous or exogenous in origin. Endogenous hormones are more common and typically result from ovarian remnant syndrome.[51-55] Regardless of the source of the hormones, the effect on the uterus is the same. Cystic endometrial hyperplasia, with or without and endometritis, initially develops. Bacteria from the vagina or via hematogenous infection then invade the uterine lumen and proliferate resulting in pyometra.[51,52,54,55] A uterus previously primed with cystic endometrial hyperplasia and/or endometritis may develop pyometra weeks to years later even without additional hormone exposure, although in many cases of stump pyometra, there is either an endogenous (ovarian remnant or adrenal) or exogenous progestagen exposure.[51,52,54,55] Abscessation may also occur as a result of suture reaction, use of non- absorbable suture in the uterine body ligatures, excess tissue manipulation causing tissue trauma, prior endometritis or pyometritis at the time of ovariohysterectomy, or failure to use aseptic surgical technique.[51,53] In some cases, suture reaction will occur resulting in

granuloma formation. Use of non-absorbable suture in particular, will cause more suture reaction than absorbable suture material. Suture material can also be carcinogenic and initiate sarcomas.[56]

It is common practice, particularly in young patients, to leave all or part of the uterine body and often part of the uterine horns during a spay because the surgeon tries to use as small an incision as possible. Leaving all or part of the uterus predisposes the patient to stump pyometra later in life if an ovarian remnant is present or the animal is exposed to exogenous steroids. While the main concern about leaving uterine tissue at the time of spay is stump pyometra or stump granuloma, neoplasia can also develop in the uterine stump. While leiomyoma, leiomyosarcoma and lymphosarcoma are common neoplasms of the canine uterus, hemangiosarcoma may also develop in the uterus and present as bloody vulvar discharge.[57,58]

Clinical signs include all those typically associated with pyometra, including depression, anorexia, fever, inappetance, vomiting, abdominal pain or distension, polyuria, polydipsia, with or without vaginal discharge.[50,51,53] There may be leukocytosis with a left shift, monocytosis, azotemia, elevation of hepatic enzymes, hypoalbuminemia, hyperglobulinemia, non-regenerative anemia.[50,51,53] Stump granuloma may present with urinary signs (stranguria, pollakiuria, hematuria, dysuria, incontinence), digestive signs (dyschezia, constipation, incontinence), signs of bloody vulvar discharge, or the lesion may be palpable as an abdominal mass in the caudal abdomen on physical examination.

Surgical treatment is necessary.[50-54] The affected stump tissue should be removed whenever possible. Adhesions may involve the urinary tract, body wall or pelvic canal. Surgical complications include septic peritonitis, septicemia, ureteral or bladder trauma, incontinence, suture reaction and dehiscence.[50-53] Both ovarian pedicles should be examined and any granulation tissue or ovarian tissue at these sites should be removed and examined histologically.[50-53] If the stump tissue cannot be completely removed because of extensive adhesions, options include suctioning or aspiration of infected contents, drain placement, marsupialization or omentalization.[51,53] Aspiration should not be used if the patient is septic or has peritonitis or has a source of continued infection.[51,53]

This disease can be completely prevented by complete removal of the entire uterus, ligating the uterine body at the level of the cervix.[50]

Vaginal masses

Vaginal masses include vaginal prolapse, neoplasms of the vagina and urethra, clitoral hypertrophy, abscesses, hematomas or cystic structures[59-67] Vaginal prolapse, also called vaginal hyperplasia/hypertrophy, is typically a disease of intact bitches, but spayed bitches may develop vaginal prolapse if they have an ovarian remnant; are exposed to exogenous estrogens via foods or supplements; ingest estrogenic medications (for urinary incontinence or a human medication) or are exposed to a estrogen cream or patch used by their owner. Vaginal prolapse occurs due to edema of the submucosa and hypertrophy of the stratified squamous epithelium resulting in a protrusion of a mass of round tissue from the floor of the vagina or doughnut shaped tissue protruding circumferentially from the walls of the vagina.[59] Bitches may lick excessively at the prolapsed tissue or show signs of stranguria or dysuria.[59] Spontaneous regression occurs with removal of the source of estrogen.[59] Surgical removal is typically not necessary in spayed bitches if the source of estrogen can be identified and removed.

Vaginal tumors account for 2.4-3% of tumors in dogs.[66,68,69] Excluding transmissible venereal tumors (TVT), 81-83% are reported to be benign.[68-70] The most common benign tumors are leiomyomas, fibromas, polyps and lipomas.[62,63,65,66,68-71] Extraluminal leiomyomas usually arise from the roof of the vestibule, while intraluminal leiomyomas typically arise from the vestibular wall and can become quite large (10-12 cm).[59,70] The most common malignant tumors are leiomyosarcoma and squamous cell carcinoma.[66,68] Botyroid rhabdomyosarcoma,[72] hemangioma,[67] hemangiosarcoma,[73] lymphangiosarcoma,[74] neurofibroma, histiocytoma, mxyoma, myxofibroma, and mast cell tumors[75] have also been described. The mean age of occurrence is 10+ years.[59,69,70] Malignant tumors have been reported with higher frequency in spayed bitches.[69] Some of these tumors may be hormone responsive, so the owners should always be queried about exogenous hormone exposure if the patient has been ovariectomized.[62,65,68-70,76]

Clinical signs usually include excessive licking, protrusion of the mass through the vulvar lips or perineal swelling, hemorrhagic or mucopurulent vulvar discharge, stranguria, dysuria, hematuria, pollakiuria, incontinence, tenesmus or dyschezia.[59-65,68,69] Benign tumors tend to be more pedunculated, while malignant tumors tend to more invasive and are therefore more difficult to excise.[59,60,62,65,68-70]

Treatment includes surgical resection, laser ablation, chemotherapy, or radiation therapy depending on location.[60,62,64,65,68,70,73,76-78] Vaginal cysts may be marsupialized into the vaginal canal to allow continued drainage or they can be surgically removed.[60,61,64] Some masses may require episiotomy, vulvovaginectomy and perineal urethrostomy for removal.[75-77] Tumors arising from the anterior vagina may require vaginectomy with pubic and ischial osteotomy.[77] Use of aglepristone, a progesterone receptor antagonist, may be beneficial to reduce the size of hormone responsive tumors, prior to surgical excision.[79]

Urethral neoplasia may be epithelial (transitional cell carcinoma and squamous cell carcinoma) or mesenchymal (leiomyoma, leiomyosarcoma).[59] Most of these tumors are malignant.[59] The mean age of occurrence is 10.2 years.[59] Clinical signs include stranguria, hematuria, dysuria and bloody vulvar discharge.[59]

Transmissible venereal tumor is typically a neoplasm of intact animals, but if an animal with a small tumor was spayed, it may not be diagnosed until vaginal discharge developed days to months later . Since this tumor is spread during coitus it is typically acquired when the bitch is intact,[68] however, on occasion, spayed bitches will stand to be mated by a male dog. Bitches with ovarian remnants may also acquire TVT when the remnant becomes steroidogenically active and estrus behavior returns. Clinical signs with TVT usually include serosanguinous vulvar discharge.[59,68,69] The mass may have multiple nodules and is typically proliferative in appearance (cauliflower-like) with a friable surface.[59,68,69] Either vincristine alone or combined with surgical excision is the treatment of choice; while radiation therapy may also be effective.[59,68,69]

Clitoral enlargement may occur in spayed animals as a result of endogenous steroid production from the adrenal glands, exogenous androgen or anabolic steroid administration, accidental exposure or exposure to an owner's testosterone cream or patch.[59] Clitoritis or vaginitis may result from exposure or from trauma caused by licking.[59] Removal of the source of steroid may result in resolution of signs with no further treatment.[59] Typically, if there is no os clitoris, the hypertrophy will resolve with cessation of hormone exposure.[59] Surgical removal is possible if hypertrophy persists.[59]

Summary

Reproductive disorders in previously spayed bitches are common. They can be very frustrating cases for the veterinarian and the owner alike. A step-wise approach to diagnosis is necessary and may require time and a variety of testing to arrive at a concrete diagnosis. Once a diagnosis is made, treatments are relatively straightforward. Chronic vaginitis cases are the most frustrating as they may never completely resolve and may require lifelong therapy.

Table 1. Foods with potentially high estrogen content

Alfalfa	Eggplant	Pumpkin
Animal Flesh	Fennel	Red beans
Anise Seed	Flaxseeds	Red clover
Apples	Garlic	Rhubarb
Baker's yeast	Hops	Rice
Barley	Licorice	Sage
Beets	Oats	Sesame seeds
Carrots	Olive oil	Soybean sprouts
Cherries	Olives	Soybeans
Chickpeas (garbanzo beans)	Papaya	Split peas
Clover	Parsley	Sunflower seeds
Cowpeas (black-eyed peas)	Peas	Tomatoes
Cucumbers	Peppers	Wheat
Dairy foods	Plums	Yams
Dates	Pomegranates	
Eggs	Potatoes	

From: http://www.holisticonline.com/Remedies/hrt/hrt_food_and_estrogen.htm

Lignans (most fruits and vegetables) are weak estrogens that block the action of other estrogens and isoflavones (soy, dairy, gluten grains and legumes) stimulate estrogen receptors.

Table 2. Bacterial isolates from the vaginal canal of healthy and diseased, intact and ovariectomized bitches

	Hirsh[19]	Bjurström[20]	Olson[23]	Doig[24]	Bjurström[26]
Mycoplasma spp		59.3%		88-95%	
Ureaplasma spp				37-50%	
Escherichia coli	31-45%	84.7%	38-45%		32.1%
Staphylocci spp	13-27%	33.9%	23.8 - 66.7%		12.8%
Streptococci spp	43-61%	47.5-55.9%	9.5-28%		19.2%
Proteus spp	7-13%	25.4%	4.8-15%		6.4%
Bacillus spp	2-3%		14.3-15%		
Corynebacterium spp	7-11%	40.7%	9.5-10%		
Pseudomonas spp	4-9%	10.2%	5%		
Micrococcus spp			14.3%		
Neisseria spp			4.8%		
Klebsiella spp	1%		4.8%		
Hemophilus spp	1%				
Pasteurella spp	5-26%	98.3%			6.4%
Enterococcus spp	2%	44.1%			1.3%
Moraxilla spp	2%				
Flavobacterium	3%				

*Percentages indicate the percentage of individuals where the organism was recovered.

Table 3. Progesterone concentrations in intact anestrus bitch and ovariectomized bitches before and after stimulation with cosyntropin[40]

	Range	Average
Intact basal	0.01 – 0.65 ng/ml	0.21 ng/ml
Intact post-stimulation	0.3 – 3.7 ng/ml	1.7 ng/ml
Spayed basal	0.01 – 0.14 ng/ml	0.04 ng/ml
Spayed post-stimulation	0.3 – 1.3 ng/ml	0.8 ng/ml

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Evaluation of the tom: collection procedures, evaluation of sperm, and subsequent use

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Abstract

When managing catteries, it is important to understand appropriate behavior and management of the tom cat in order to diagnose and manage fertility problems. Semen collection and subsequent use in the domestic cat has its own unique challenges, but is important for analysis of fertility and the implementation of advanced reproductive techniques. Improved techniques for the collection and use of feline spermatozoa is vital as it allows the preservation of valuable genetics in pure breed catteries, and preservation of rare and endangered feline species. The domestic cat also serves as an important model for several human diseases and preservation and maintenance of these genetics is necessary. The intent of this review is to offer a detailed protocol for the successful collection, evaluation, and use of feline sperm.

Keywords: Feline, cat, tom, sperm collection, electroejaculation

Introduction

Interest in the domestic cat examination, semen collection, evaluation, and use has increased in the past ten years. Because the domestic cat can serve as a model for reproduction of exotic and endangered feline species, and as an animal model for human disease, research interest in assisted reproduction has increased. Additionally, preserving the valuable genetics in pure breed catteries has also increased the demand for quality reproductive management. Semen collection and evaluation is vital to diagnose and monitor progress for any reproductive problem. However, the process presents its own set of challenges unique to the cat due to the small volume and comparatively low sperm number produced in each ejaculate as well as the decreased overall sperm quality seen in many exotic species or research catteries. The demand for semen collection, evaluation, and subsequent use is growing as a way to preserve important or valuable genetic materials.

History

A complete reproductive and general management history is extremely important when evaluating the tom for infertility, as many of the issues may be management practices. Questions should include (but not limited to) type of housing, mating practices, time with female, number of matings observed, previous litters sired, litter size, and any previous sperm analysis performed. Also important to document is if the female shows the typical after reaction following mating. If this does not occur, the tom may not be achieving vaginal penetration of the queen and therefore not inducing ovulation or ejaculating within the vaginal vault.

Examination

In addition to a complete history, a general physical examination should be performed, possibly with a complete blood count and serum chemistry to evaluate the overall health of the animal. A reproductive examination should consist of palpation of the testicles, assessing for size, texture, and symmetry followed by ultrasound to assess any irregularities within the testicular parenchyma. The penis should also be evaluated for any discoloration, discharge, and the presence of spines. Exteriorization of the penis may be difficult in the unsedated animal, so this portion of the examination might be best accomplished just prior to sperm collection when the tom is sedated or anesthetized.

Semen collection methods

The artificial vagina (AV) for the domestic cat is most commonly constructed using an Eppendorf tube and a rubber pipette bulb. The male is allowed to mount a queen, and the AV is held in place to facilitate and collect the ejaculate. If the male has been adequately trained to the AV, or a queen in estrus is not available, the male may be allowed to mount a gloved arm. The advantages of using an AV are that

semen collection can be performed readily in the unanesthetized tom and allows a complete ejaculate to be obtained. Disadvantages are the requirement for training and a frequent necessity of a teaser queen. Often, weeks to months of conditioning and training are required before a tom is consistently producing ejaculates, and training may not be successful in all toms. Valiente et al worked with toms in their cattery three times weekly for 20 minutes and reported a mean of 3.9 months (1.5-5.5 months) before the first ejaculate was produced.[1] With patience, collection using an AV is an excellent method for situations where a single male or group of toms are collected on a regular basis, such as in a cattery or research colony, but is impractical for a single evaluation in a clinical setting using an untrained tom.

Elecroejaculation has been the most common method of obtaining an ejaculate from a tom that is not trained to an AV. The procedure requires general anesthesia. This author's preferred anesthesia protocol includes dexmedetomidine (30-40 μg/kg) and ketamine (3-5 mg/kg) in the muscle, followed by intubation and supplemental oxygen. Inhalant anesthesia (isoflurane) can be added if necessary, but the short procedure time generally does not require it. On occasion, electroejaculation using inhalation anesthetics may result in urination and contamination of semen samples. Zambelli et al compared the use of medetomidine alone to ketamine alone on the quality of ejaculates collected by electroejaculation. These researchers found that the use of an α_2 agonist (medetomidine; Domitor, Pfizer, Florham Park, NJ) produced higher numbers of spermatozoa in the ejaculate than using ketamine alone, and did not increase the incidence of retroejaculation.[2] However, these researchers did not evaluate the use of these medications in combination. To prevent any perception of discomfort during the procedure, it is recommended that an anesthetic, such as ketamine, be added to balance the sedative and analgesic effects of dexmedetomidine. Each tom should be monitored appropriately while under general anesthesia to minimize anesthetic complications.

Procedure for electroejaculation

- Electroejaculation is performed using a rectal probe one cm in diameter and 12-13 cm long. Appropriate electroejaculators are available commercially (P-T Electronics, Boring, OR).
- Lubricate the rectal probe with non-spermicidal lubricant and insert the probe gently into the rectum approximately 5-7 cm. The electrodes should be oriented ventrally.
- If feces in the rectum prevent the placement of the probe, a lubricated gloved finger may be used to evacuate the rectum but is not always necessary.
- Manually extend the penis and clean with gauze moistened with saline (no alcohol or soap). Dry the penis with clean or sterile dry gauze.
- Place a sterile vial (Eppendorf) over the penis. Alternatively, one could use a sterile 5 ml sample collection vial but the small volume of ejaculate may be more difficult to manage in a larger vial.
- Turn on the ejaculator. Make sure the rheostat dial is set to zero prior to activating the power switch.
- Rotate the rheostat to provide a series of electrical stimuli by turning the dial to the desired voltage for 2-3 seconds, then abruptly back to zero for 2-3 seconds. The stimuli should be administered in the following order:
  - Set one: 10 times with 2 volts, 10 times with 3 volts, 10 times with 4 volts, rest 3-5 min.
  - Set two: 10 times with 3 volts, 10 times with 4 volts, 10 times with 5 volts, rest 3-5 min
  - Set 3: 10 times with 4 volts, 10 times with 5 volts, 10 times with 5 volts (or 6 if needed, depending on previous response).
- The sample obtained between each electroejaculation set should be evaluated for the presence of sperm. This is usually readily evident, as an ejaculate containing spermatozoa will be cloudy. A new, sterile tube should be used between each set after collection to prevent contamination or loss of the sample.

The tom's response to the stimuli should be monitored and the probe location be adjusted accordingly. During the stimulation, both hind limbs typically would extend symmetrically. If they are not extending, or if one extends more than the other, confirm that the probe is in contact with the rectal wall, and that the electrodes are on ventral midline.

Because electroejaculation requires specialized equipment, collection using urethral catheterization has become very popular as a means to procure a sample for semen analysis. Following heavy sedation with medetomidine (130-140 µg/kg, IM), collection of semen was successful by urethral catheterization. A comparable dose of dexmedetomadine (Dexdomitor, Zoetis) would be 65-70 µg/kg IM. Once heavy sedation is achieved, the penis is exposed and cleaned with saline. A tomcat urinary catheter (open ended) is inserted into the urethra approximately 8-9 cm and removed. Care must be taken not to enter the bladder with the catheter. Once removed, the catheter can be flushed with appropriate extender and the sample evaluated. Although the total sperm collected was lower than what was obtained by electroejaculation, Zambelli et al were able to collect adequate numbers of spermatozoa for insemination or crypreservation.[3] This provides an excellent alternative method for semen collection in a practice setting.

As an alternative to an ejaculate, sperm collection by epididymal flushing after castration or postmortem followed by cryopreservation has been widely described.[4-6] The research performed in the domestic cat has provided a model for the technique in endangered feline species in attempts to preserve valuable genetics.[7] A recent report showed that sperm motility and membrane integrity was decreased when the cat was euthanized with pentobarbital prior to epididymis collection when compared to induction of general anesthesia.[8] Therefore, if an animal is undergoing epididymal sperm collection followed by euthanasia, it is advised to anesthetize the cat for castration, and then administer the euthanasia agent after the testicles are removed. If cryopreservation is not feasible in the clinical setting, epididymal sperm recovery can be performed, processed, and cooled to 5°C, and shipped overnight to a facility for freezing. Cooling for 24 hours did not affect the post-thaw motility.[9]

Spermatozoa for morphologic evaluation may be collected by aspiration or lavage of the queen's vaginal vault following mating. Collection by this method may be useful to rule out azoospermia, but there may be a higher rate of morphological defects in this sample because normal sperm should be moving out of the vaginal vault and into the uterus very quickly following breeding. Because retroejaculation is common in the tom, cystocentesis and analysis of the urine after ejaculation may yield enough sperm cells for a limited analysis, but both sperm motility and morphology are likely to be compromised.

Semen evaluation

Following collection, the volume of the ejaculate is recorded, and the sample is extended at least 1:1 immediately (slow, drop-wise addition with mixing) with suitable media. This author prefers Ham's F-10 with 25 mM Hepes, 1mM pyruvate and glutamine, penicillin/streptomycin/ neomycin, and 5% fetal bovine serum. An alternative that is commercially available is TEST yolk buffer comprising of tes, tris lactose and 20% egg yolk, however, analysis of the motion characteristics might be difficult due to the interface egg yolk droplets if the extender is not filtered prior to use. Motility (total and progressive) should be recorded by estimation under low power microscopy on a warmed microscope slide, or using a computer assisted analysis (CASA) calibrated for feline spermatozoa. Concentration is obtained using a hemacytometer at a 1:100 dilution. The NucleoCounter® (ChemoMetric A/S, Allerød, Denmark) has also been used clinically to determine feline sperm concentration.

Evaluation of sperm morphology is an integral part of the semen evaluation. Teratospermia in the domestic cat has been defined as less than 40% morphological normal sperm.[10] There is a very high degree of teratospermia in many of the exotic feline species studied, complicating genetic preservation.[11] Teratospermia is also observed in small populations of cats where inbreeding has occurred.[11] Research has shown that a single generation of inbreeding (offspring bred to parent) produced male offspring with less than 15% morphologically normal sperm compared to 55% morphologically normal sperm in control animals, indicating that loss of genetic diversity leads to increased teratospermia in as little as one

generation.[11] The degree of teratospermia and the nature of the defects present affect the post-thaw survival of cryopreserved sperm, and the freezing method may need to be adjusted to compensate for these defects. For example, rapid cooling of sperm from teratospermic cats results in a large number of damaged acrosomes compared to normal controls. This damage is decreased when using a slower cooling rate.[12]

In other species, the number of morphologically normal sperm and motility of the sperm are closely correlated. In the cat, many teratospermic ejaculates demonstrate adequate motility (greater than 70%), in spite of low number of morphologically normal sperm. Other measured parameters of the ejaculate may include pH, osmolality, membrane integrity, sperm chromatin structure, bacterial culture, or seminal plasma chemistry.

The ejaculate characteristics expected for each collection method can be found in the Table. During natural matings, copulation occurs multiple times within a short period of time. This is necessary for the induction of ovulation. A suggested breeding dose using vaginal artificial insemination is $80x10^6$ spermatozoa (motility greater than 80%) and achieved a pregnancy rate of 77.8%. Vaginal insemination with $20x10^6$ and $40x10^6$ obtained a conception rate of 6.6% and 33.3%, respectively, in that same report.[13] If collecting via AV, two collections, 10-15 minutes apart should produce sufficient numbers.[13,14]

Use of semen

Once the ejaculate is collected and the volume is measured, this author extends it immediately 1:1 to 1:3 with Ham's F-10 with 25 mM Hepes, 1mM pyruvate and glutamine, penicillin/streptomycin/ neomycin, and 5% fetal bovine serum. This extends the volume of the sample to make it easier to work with. Sperm collected following each ejaculation set is extended, and then all samples are combined at the end to make up the total ejaculate. In this author's experience, the total volume of the ejaculate (without extender) obtained using electroejaculation ranges from 50-130µl (average 94.1µl). Once the motility and morphology are evaluated, the ejaculate can be centrifuged (300 x g for 8 minutes) if a smaller volume is needed for intrauterine insemination, or vaginally inseminated into an estral queen that has received ovulating induction agents. If cooling and transporting is necessary, the ejaculate can be extended with refrigeration medium component (TEST-Refrigeration Medium; Irvine Scientific, Santa Ana, CA) and cooled to 5°C for holding overnight or shipping. In this author's experience, extending the ejaculate to a concentration of 30 million sperm per ml in TEST Refrigeration Medium extender produced acceptable motility characteristics after 24 hours at 5°C.

Conclusion

The amount of scientific information involving the domestic cat has greatly increased making the diagnosis, treatment, and management of fertility issues a reality in the tom. With improved semen collection techniques, complete evaluation of the spermatozoa is possible, as is the subsequent insemination of the sperm into a female to achieve pregnancies.

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Sperm Parameter	**Artificial Vagina**	**Electroejaculation**	**Catheterization**	**Epididymal Collection**
Volume (µl)	102.6 +/- 14[‡] 42.8 +/-5.6[§]	67.1 +/- 25.9[*] 94.1 +/- 40[π]	10.5 +/- 5.3[*]	variable
Concentration (10^6/mL)		542.9 +/- 557.9[*]	1868.4 +/- 999.8[*]	variable
Total Sperm Number (10^6)	83+/-0.4[§]	33.6 +/- 34.5[*]	21.0 +/- 18.1[*]	variable
Total Motility (%)	90.9 +/- 1[‡] 92+/-1.1[α]	78.1 +/- 10.3[*]	50.4 +/- 20.3† 78.1 +/- 9.6[*]	71.5 +/- 9[†]
Progressive motility (%)	88.6 +/- 0.9[‡]	90[*] 75+/-5[¶]	30.5 +/- 18.3[†]	50 +/- 12.4[†]
Morphologically Normal Sperm (%)	90[‡]	25+/-10[¶]	41.5 +/- 18.5[†]	58.1 +/- 16.6[†]

Table: Sperm characteristics for various collection methods in the tomcat.

[*] Zambelli et al. (2008)[3]
[†] Filliers et al (2010)[15]
[‡] Valiente et al (2014)[1]
[§] Oba et al (2011)[14]
[¶] Johnson, unpublished

Evaluation and insemination options for the subfertile queen

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Abstract

Causes of infertility in the queen are often multifactorial. Cattery management should be addressed prior to evaluation of individual animals. Factors such as housing/lighting, preventive care, and retroviral disease status of the colony should be evaluated. A complete physical examination is vital to rule in or out anatomical abnormalities leading to infertility. Transabdominal ultrasound is a useful tool for noninvasive evaluation of the reproductive tract, and vaginal cytology combined with hormone assay and behavior can be used to stage the estrous cycle. In queens with good breeding management, cystic endometrial hyperplasia is a common cause of infertility and is a major predisposing factor for pyometra. Recent research has demonstrated continued advances in assisted reproductive techniques in felids, including several options for the use of frozen semen.

Keywords: Queen, cat, infertility, pyometra, artificial insemination

Introduction

The female cat is a seasonal polyestrus induced ovulator, and appropriate breeding management is often the key to good fertility in a cattery. Minor changes in procedures may have an enormous impact on pregnancy rate and litter size. When presented with an infertile queen or generalized infertility in a cattery, a detailed history and observation of protocol is as essential as the complete physical examination. The queen is a long day breeder, therefore records of the animals should be evaluated to see if the infertility is seasonal. Maintaining the animals under artificial lighting for at least 14 hours per day reduces seasonal variation, but in this author's experience, even cats maintained under this lighting system experience a decrease in pregnancy rates during the shorter days of the year. A queen will typically show estrus within one to two months after initiating an artificial lighting period.

The queen requires more than one mating to achieve enough stimulation for the luteinizing hormone (LH) surge necessary for ovulation. Ovulation occurs in approximately 50% of queens following one mating, but approaches 100% of queens allowed to mate four or more times.[1] Observation of breeding episodes is helpful to evaluate the number of times the tom attempts to copulate and also evaluating the female for the classic "after reaction" that confirms copulation took place. If the after reaction does not occur, penetration of the penis into the vagina was unlikely.

A general and reproductive physical examination (with blood chemistries and endocrine testing when indicated) should be performed to rule out obvious causes of infertility dealing with physical limitations (vaginal stricture) or endocrine abnormalities. If physical abnormalities are present or if there is no other obvious cause for the infertility, a karyotype of one or both members of the breeding pair may be necessary to confirm the genetic makeup of the animal is normal.

Use of a high quality ultrasound unit will allow imaging of the uterus by starting at the bladder and moving cranially. The uterus should be evaluated for size, symmetry of the horns, the presence of cystic structures and fluid within the lumen. Ovaries can usually be detected immediately caudal to the kidneys and can be evaluated for the presence of large follicular cysts.[2]

Management

Successful management of a breeding cattery requires knowledge of normal feline breeding behavior. General management practices to ensure cleanliness of facilities and animals, comfortable housing, and adequate ventilation are required. The vaccination status of the animals should be evaluated to ensure adequate disease control. While a closed colony is preferable with no new animals entering, such a policy may also limit the introduction of new genetics. A quarantine period with adequate testing procedures should be mandatory for all new additions.

When breeding naturally in a cattery, the queen should be taken to the tom, preferably when she is showing signs of estrus (heat). Signs include vocalization, rubbing on walls, and rolling on the floor. Additionally, vaginal cytology examination may be performed every two to three days. A moistened sterile cotton tipped applicator is inserted gently into the vaginal canal of the cat approximately 1cm. Vaginal cytology can be assessed for the percentage of cornified cells. In this author's experience, the percentage of anuclear cornification during estrus is lower in the queen than in the bitch and many queens will be receptive to the male when the vaginal cytology is 70-75% cornified. Inducing ovulation by taking a vaginal cytology is a concern for some practitioners, but if the sample is taken gently, the risk is low because LH release requires multiple mating episodes over a short period of time. A single swab is unlikely to be enough stimulation to induce the LH surge.

If heat detection is not possible, the queen can be placed with the male for at least two weeks. The pair should be monitored closely for breeding activity or fighting (if the queen is not in estrus). If fighting occurs, separate the pair and retry in a few days, or until the vaginal cytology indicates estrus. Once the pair has been together for at least two weeks, serum progesterone concentration can be used to confirm ovulation (greater than 2 ng/ml). At this time, the queen may be returned to her housing awaiting a pregnancy examination. If the progesterone is less than 2 ng/ml, mating has likely not occurred, or has not occurred sufficiently to induce the LH surge and ovulation. The queen should be returned to the male and monitored for breeding activity. If ovulation still does not occur, inducing ovulation followed by artificial insemination may be necessary.

Although cats are induced ovulators, spontaneous ovulations sometimes occur. If a female continues to refuse a male, a serum progesterone concentration should be assessed to determine of luteal tissue is present. If the progesterone is greater than 2ng/ml, luteal tissue is present. In the non-pregnant queen, the luteal phase lasts approximately 45 days, and the queen should be monitored every one to two weeks utilizing vaginal cytology, behavior, or progesterone assay to determine when the luteal phase has ended and routine breeding management instituted at that time. Pregnant queens should be placed in a quiet area, alone if possible, with a covered box for nesting and queening. A stressed queen is more likely to cannibalize her young so every attempt should be made to reduce stress around the time of queening.

Causes of infertility

Causes of infertility in the queen are often divided into broad categories but this discussion will focus on those that fail to produce a pregnancy despite appropriate breeding management. In a natural breeding situation, queens that show persistent receptivity to the male are likely failing to ovulate. This may result from lack of adequate stimulation to produce the LH surge or a primary ovarian dysfunction such as ovarian follicular cysts. Evaluation of the breeding records, number of witnessed matings, and presences or absence of the "after reaction" of the queen following the coitus will assist in assessing if adequate stimulation has occurred. If ovulation is not occurring due to inadequate copulatory stimuluation ovulation may be induced pharmacologically (see below) and followed by either natural mating or artificial insemination. The formation of ovarian follicular cysts can be a frequent occurrence in the queen and incidence increases with age. Presence of follicular cysts is best confirmed by transabdominal ultrasonography.[2] Cysts are often hormonally functional, producing high concentrations of estrogens. Treatment options include attempts to induce luteinization of the cyst(s) by administration of either 500 IU human chorionic gonadotropin (hCG) or 25µg gonadotropin releasing hormone (GnRH) intramuscularly. Surgical resection has also been reported with a return to fertility.[3]

In this author's experience, the most common primary uterine problem in the queen is cystic endometrial hyperplasia (CEH) with or without concurrent pyometra. In the author's research cattery, approximately 75% of queens diagnosed with infertility (failure to produce offspring following appropriately managed breeding(s)) had histological evidence of CEH within their uterus. Clinical signs in this CEH affected group of queens were first noted at three years of age (decreased litter size or failure to produce kittens consistently). Intact female cats over the age of five years have been shown to be at risk for clinical disease associated with CEH/pyometra.[4] Diagnosis can be suspected based on history of reduced fertility and by the ultrasonographic appearance of the uterus in severe cases. A uterine biopsy

provides definitive diagnosis and may be required for less severe cases; typically, these are obtained surgically. If CEH is severe, prognosis for fertility is guarded and ovariohistorectomy is recommended. Pyometra often occurs secondary to CEH and, when severe, necessitates ovariohysterectomy (preferred treatment). In the author's cattery, pyometra has been observed in young queens (less than three years of age). In these cases the uterus appeared grossly otherwise healthy, lacking clinical signs of underlying CEH. The queens were not systemically ill and the only clinical sign was a purulent vaginal discharge. Treatment with enrofloxacin (5mg/kg intramuscularly or orally twice daily) and natural prostaglandin (0.25 mg/kg dinoprost tromethamine subcutaneously or intramuscularly twice daily) was followed by a return to fertility in over half the cases treated. The uterus should be monitored daily by gentle palpation and transabdominal ultrasound to assess amount of fluid within the lumen and overall uterine size during treatment. Prostaglandin therapy should be continued until fluid accumulation within the uterus is resolved and the uterus has returned to normal size. Breeding may be attempted approximately four weeks after successful treatment. In a small number of the treated queens, recurrence of the condition was observed during the next luteal phase. In these cases the histopathology demonstrated CEH which likely favored recurrence. A technique for nonsurgical uterine lavage as a treatment for uterine infection has been described in the large non-domestic, larger feline species, but would be much more difficult to perform in the smaller domestic queen.[5]

Ovulation induction and artificial insemination

Ovulation induction

Queens are induced ovulators, thus ovulation induction protocols can be critical to success. One group has shown consistent results inducing estrus and ovulation using a combination of equine chorionic gonadotropin (eCG) and hCG in a timed artificial insemination protocol.[6] Treatment is initiated in nonestral, nonluteal queens as determined by observing for behavioral estrus and serum progesterone less than 1 ng/ml. Queens received an initial injection of eCG (100 IU IM) followed by an injection of hCG (75 IU IM) 85 hours later. Insemination is performed 31-33 hours after the hCG injection and ovulation is expected between 25-30 hours after hCG. Using this protocol, 100% of queens ovulated with a 75% pregnancy rate (6/8 queens) when inseminated with either laparoscopic intrauterine or laparoscopic oviductal inseminations.[6] An alternate method is to administer hCG intravenously twice daily on days two through four of estrus. The ovulation rate using this protocol was 95.6% (43/45 queens).[7] In this study, queens were inseminated at 15, 20, and 30 hours following the last hCG injection. Ovulation is expected to occur between 25-27 hours after administration of hCG.

One concern with insemination protocols is the effect of anesthesia on ovulation. Howard, et al reported that queens inseminated (laparoscopic intrauterine) after ovulation produced more corpora lutea and embryos, and had a higher pregnancy rate than those inseminated prior to ovulation (50% vs 14.3% pregnancy rate). A mean of 6.6×10^6 motile sperm were used in both groups.[8] Due to the concern that anesthesia may inhibit ovulation, many researchers elect to inseminate after ovulation has occurred (28-40 hours). However, others have not found an effect of anesthesia on ovulation rate with slightly higher doses of hCG.[7]

Vaginal insemination

When frozen semen was used for vaginal insemination, the pregnancy rate was low (10.7% or 6/56 attempts).[9] Vaginal insemination is therefore best reserved for cases in which fresh or good quality cooled transported semen is available. This procedure may be performed by inserting an open ended tomcat catheter approximately 1-2 cm into the vaginal vault in the non-anesthetized queen, depositing the semen, and elevating the hind quarters for approximately ten minutes to prevent back-flow. This technique is difficult in many queens due to a less than compliant nature and the violent after reaction associated with breeding/vaginal stimulation. Most researchers have better success using a deep vaginal insemination technique under general anesthesia. In the anesthetized queen, the vaginal vault is initially dilated with a 2mm diameter probe. The insemination pipette is then inserted 3-4 cm into the vaginal

canal, the semen is deposited and the hind quarters elevated after insemination. Ovulation induction is still required following transvaginal insemination regardless of whether or not anesthesia was used. The pregnancy rate following vaginal insemination was 77.8% when 80×10^6 fresh motile sperm were used. The pregnancy rate decreased to 33.3% using 40×10^6 motile spermatozoa and 6.6% with 20×10^6 motile spermatozoa. The use of at least 80×10^6 motile spermatozoa be used when performing a vaginal insemination is commonly recommended.[7]

Intrauterine insemination

The main advantage of intrauterine insemination in the queen is the reduction in insemination dose compared to vaginal insemination. In fact, a pregnancy rate of 80% was achieved using only 8×10^6 motile sperm (10% of the recommended vaginal dose) when inseminated surgically into one uterine horn [10] and lower doses have also been successful. Intrauterine insemination is performed surgically with the queen under general anesthesia. The standard surgical method is to make a midline incision on the ventral abdomen. Both uterine horns are located and examined for signs of pathology prior to insemination. Following visual examination, one uterine horn is isolated. A 22-20ga intravenous catheter is inserted through the uterine wall (mid-horn) and into the lumen. The catheter is advanced off the needle into the uterine horn in the direction of the ovary. The needle is removed. Half of the semen (approximately 10µl) is infused through the catheter and into the uterine horn. Care should be taken not to contaminate the abdomen with the sperm sample. Once insemination is complete, the catheter should be removed and a gauze pad should be placed with direct pressure over the insertion site to control bleeding. The process is repeated using the remaining semen into the opposite uterine horn with a fresh catheter.

Alternatively, the insemination may be performed laparoscopically.[6] The uterus is held adjacent to the body wall using atraumatic forceps. An 18ga catheter is inserted through the ventral abdominal wall and into the uterine lumen as described above. Polyethylene tubing was then inserted through the catheter and into the uterine lumen. The semen was infused into the uterus through the tubing. When using very small amounts of semen, placing the sample in the distal end of the tubing will minimize loss of the inseminate.

Oviductal insemination

Recently, success has been described using very low numbers of sperm laparoscopically inseminated directly into the oviduct. This method is desirable in species or situations where the number of available sperm is very low. Swanson's group has shown excellent success using as few as one million motile sperm. This technique requires the use of custom made forceps to grasp and secure the ovarian bursa. The bursa is then manipulated until the abdominal oviductal ostium is visualized. An 18ga intravenous catheter is inserted through the abdominal wall to serve as a port. A modified blunted 22ga needle with attached syringe is then inserted through the catheter and into the oviductal ostium (approximately 2 cm) and 5µl of semen (1×10^6 motile sperm) is infused into the oviductal lumen. One study found a pregnancy rate of 45% with a greater litter size when compared to laparoscopic intrauterine insemination (pregnancy rate of 18%) with the same insemination dose.[6]

Conclusion

In conclusion, breeding management of the subfertile queen may require veterinary intervention with one of the described artificial insemination techniques. Management of the male and female, ensuring adequate sperm for insemination and induction of ovulation is vital. However, in many cases, success can be found with persistence and practice.

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Effect of antibacterial agents in semen extender on bacterial growth in extended canine semen held at 5°C and 20°C for up to 48 hours

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Abstract

It has long been believed by some theriogenologists and dog breeders that the antibiotics in commercial semen extenders would control the growth of bacteria introduced with the semen sample. However, manufacturers include those antibiotics to prolong the product shelf life, not for the inhibition of bacterial growth. We hypothesized that the growth of aerobic, anaerobic and *Mycoplasma* species will be controlled in semen extended with commercial canine extender when held at refrigerator (5°C) or room (20°C) temperatures for up to 48 hours. Semen was collected by manual ejaculation from 14 dogs and the semen split into 11 aliquots. One aliquot each was submitted for aerobic, anaerobic, and mycoplasma cultures. Half of the remaining semen from each dog was extended 1:1 by volume with Fresh Express® (Synbiotics, Kansas City, MO) and the other half was extended 1:1 by volume with CaniPro™ ApX2 Chill 5 (Minitube, Verona, WI). The extended semen was submitted for all three cultures immediately after addition of extender, and at 24 and at 48 hours after extension; half of the semen in each extender was held at 5°C and the rest at 20°C. Bacterial growth was compared between extenders, between storage temperatures, and over time. There was no significant growth in any sample held at 5°C. There was bacterial growth in some samples held at room temperature. Percentage progressively motile spermatozoa in a given sample is not a reflection of bacterial growth in the sample.

Introduction

Biosecurity is a growing social concern. While there is a significant body of research and there are strong regulatory controls for handling, storage, and transport of semen from large animal species, there is little in the veterinary literature specifically about biosecurity concerns for breeding dogs and there are no regulations governing movement of canine semen within the United States.

Semen cannot be collected by manual ejaculation without bacterial contamination due to presence of normal bacterial flora on the urethral mucosa. Organisms also can be shed into semen as a component of prostatic or testicular fluid or by hematogenous spread from systemic infection. Not all organisms that may infect the testes may pass into semen and not all organisms shed into semen remain viable in semen. Semen extenders are liquid media that support spermatozoa through changes in temperature and provide nutrients and buffering capacity to offset acid-base changes as the spermatozoa undergo normal metabolism during storage.

Antibiotics are added to semen extenders to maintain the shelf-life of the product and are not intended by the manufacturer to control bacterial growth. In stallions, it has been demonstrated that addition of gentamicin, polymyxin B, amikacin, streptomycin, or potassium penicillin were effective at controlling growth of aerobic bacteria in semen held at refrigerator temperature but not in semen held at room temperature.[1,2] Studies specifically looking at *Mycoplasma* species in bulls and stud dogs have shown ability of extenders containing a mixture of tylosin, gentamycin, lincomycin, and spectinomycin to decrease but not eradicate growth of *Mycoplasma* over 72 hours in semen held at 5°C.[3,4] In a study evaluating growth of *Brucella canis* added to extended semen with or without additional antibiotics, it was demonstrated that bacterial growth was not negated by presence of antibiotics.[5] Veterinarians and dog owners should not assume that the antibiotics in semen extender can control bacterial growth, even in samples that are handled properly during shipment.

Significant growth of aerobic bacteria from semen is defined as growth of greater than 10^5 CFU/ml. Organisms most commonly cultured from semen from populations of normal dogs and dogs with history of reproductive tract disease were beta-hemolytic *Streptococcus* sp., *Pasteurella multocida*, beta-hemolytic *E. coli*, non-hemolytic *E. coli*, beta-*Streptococcus* sp., *Achromobacter xylosoxidans*, *Actinomyces pyogenes*, *Bacillus* sp., coagulase-positive *Staphylococcus* sp., *Hemophilus hemoglobinophilus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and

Staphylococcus intermedius).[6-8] In one study, 28.4% of samples from dogs presented for breeding soundness examination (n=39), infertility (n=25), or reproductive tract disease (n=30) yielded significant growth of aerobic bacteria.[6] In a longitudinal study of 15 fertile male dogs, 31.2% of samples yielded significant growth of aerobic bacteria. Transfer of bacteria to bitches at the time of copulation was reported in the latter study.[8]

Any growth of anaerobic bacteria from semen is considered significant. In one study, there was significant growth of anaerobic bacteria from 13.7% of samples submitted from 95 dogs, with the most common organisms identified being *Bacteroides* sp., *Peptostreptococcus* sp., *Propionibacterium acne*, *Clostridium perfringens*, *Fusobacterium necrophorum*, *Propionobacterium avidum*, and *Streptococcus morbillorum*.[6]

Mycoplasma sp. are part of the normal flora of the distal urethra in dogs. Quantitative culture is difficult so many laboratories report only positive or negative results. In one study, positive growth of *Mycoplasma* sp. was reported from 57.9% of samples submitted from 95 dogs.[6] In another study, 72% of semen samples were positive for *Mycoplasma* sp., with higher isolation rate from dogs with a history of infertility or reproductive tract disease.[9]

This study is an attempt to mimic what can happen in the real world. With semen being shipped across the country, it is imperative for the receiving veterinarian and brood bitch owner to know that the semen product they are using is safe. Shipments may have been in a hot truck all day with melted ice packs or worse yet, may be left in an uncontrolled environment. The hypothesis evaluated in this study is that growth of aerobic, anaerobic and *Mycoplasma* species will be controlled in semen extended with commercial canine extender when held at refrigerator (5°C) or room (20°C) temperatures for up to 48 hours.

Materials and methods

Male dogs were recruited for the study from local breed clubs. Semen was collected from all dogs by the authors. Only dogs that produced at least 6 ml of semen were included in the study. Males were manually collected without the use of a teaser bitch. They were collected using a latex collection device that was washed and dried in between collection of the males, and each dog's ejaculate was collected into a separate sterile centrifuge tube.

Immediately after collection, 1mL of semen was placed into anaerobic transport medium and 1mL of semen was placed into the aerobic and mycoplasma/ureaplasma transport container. These were the neat samples. The remaining volume of semen was divided in half and diluted 1:1with semen extender. Half of the semen was extended with Fresh Express® and the other half was extended with CaniPro™ ApX2 Chill 5. One mL of each extended semen sample was then submitted for anaerobic, aerobic and mycoplasma/ureaplasma. These were the Time 0 extended samples. The Fresh Express® extended semen was then divided in half. Half of the samples were held at room temperature (20°C) and the other half were stored in the refrigerator (5°C). The same was done for the CaniPro™ ApX2 Chill 5 samples. After 24 hours semen was submitted for aerobic and mycoplasma/ureaplasma from the Fresh Express® semen that was kept at 5°C and 20°C and the CaniPro™ ApX2 Chill 5 semen that was kept at 5°C and 20°C. After 48 hours semen was submitted for aerobic and mycoplasma/ureaplasma from the Fresh Express® semen that was kept at 5°C and 20°C and the CaniPro™ ApX2 Chill 5 semen that was kept at 5°C and 20°C (Figure). Because the samples would be exposed to air while keeping the samples at their appropriate temperatures, further anaerobic testing was not performed, beyond the neat and Time 0 samples, as those results would have been expected to be negative for growth. All samples were plated at a commercial laboratory using a standard three streak isolation technique and results were provided by microbiologists at that laboratory (Marshfield Laboratories, Marshfield WI).

The motility of sperm in these samples was checked two hours after collection after having been held at room temperature and after removal of the aliquots required to complete the study. Further assessment of sperm motility, at 24 and 48 hours, was performed only on the three samples that had greater than 90% motility as neat samples. All refrigerated samples were warmed on the microscope

stage for 10 seconds before evaluation. All motility assessments were made by one on of the authors (MVRK).

Results

Semen collection was attempted on 18 male dogs; semen was successfully collected from 14 of those dogs. Breeds represented were Samoyeds (n=6), Malamutes (n=6), and Springer Spaniel and Labrador retriever (n=1 each). Ages ranged from 2 years to 9 years, with a mean age of 4.5 years.

Formulations of commercial canine extenders are proprietary. Fresh Express® extender from Synbiotics corporation (www.synbiotics.com) contains several antibiotics while CaniPro™ ApX2 Chill 5 extender from Minitube (www.minitube.com) contains a single antibiotic, gentamycin. These are the two extenders most commonly used by small animal practitioners belonging to the Society for Theriogenology, as determined using an electronic survey.

The commercial laboratory reported bacterial growth as scant, light, moderate or numerous. Culture results are shown in Table 1. Overall, 35% (n=5) of the dogs used in the study had significant growth of aerobic bacteria. Thirty-five percent (n=5) of the dogs also had anaerobic growth. Seventy-eight percent (n=11) were positive for *Mycoplasma* growth and 50% (n=7) were positive for *Ureaplasma* growth at least once during the study.

Aerobic growth was considered significant if it was deemed as moderate to numerous. In the aerobic neat samples, 5 dogs had growth that was significant. The most commonly identified bacteria were *E. coli*, beta hemolytic *Streptococcus* group G, mixed urogenital/skin flora and *Enterobacter aerogenes*. Any growth of anaerobic bacteria was considered significant. In the anaerobic neat samples, there was no growth. Samples from five dogs yielded *Mycoplasma* and samples from five dogs yielded *Ureaplasma* sp.; samples from some of the dogs yielded both.

In the Time 0 samples with Fresh Express none of the dogs had aerobic growth and four dogs had anaerobic growth, with *Bacteroides fragilis*, *Clostridium perfringens* and a gram negative bacillus (most likely *Hemophilus* and *Pasteurella*), being the most commonly isolated. Samples from four dogs yielded *Mycoplasma* and samples from two dogs yielded *Ureaplasma* sp. In the Time zero samples with Chill 5 had one dog with aerobic growth, beta hemolytic *Streptococcus* group G, and two dogs had anaerobic growth with a gram negative bacillus (most likely *Hemophilus* and *Pasteurella*) and *Propionibacterium acne*. Samples from five dogs yielded *Mycoplasma* and samples from four dogs yielded *Ureaplasma* sp.

In the 24 hour samples kept under refrigeration in Fresh Express, there was no growth of aerobic or anaerobic bacteria which were cultured for separately from mycoplasma and ureaplasma organisms. Samples from two dogs yielded *Mycoplasma* and samples from five dogs yielded *Ureaplasma* sp. In the 24 hour samples kept at room temperature in Fresh Express one dog yielded *Enterococcus* species. Samples from one dog yielded *Mycoplasma* and samples from three dogs yielded *Ureaplasma* sp. In the 24 hour samples kept under refrigeration in Chill 5, there was no growth. Samples from eight dogs yielded *Mycoplasma* and samples from six dogs yielded *Ureaplasma* sp. In the 24 hour samples kept at room temperature in Chill 5, there was no growth. Samples from seven dogs grew *Mycoplasma* and samples from five dogs yielded *Ureaplasma* sp.

In the 48 hour samples kept under refrigeration in Fresh Express, there was no growth of aerobic or anaerobic bacteria which were cultured for separately from mycoplasma and ureaplasma organisms. Samples from six dogs yielded *Mycoplasma* and samples from six dogs yielded *Ureaplasma* sp. In the 48 hour samples kept at room temperature in Fresh Express, samples from two dogs had growth, with *E. coli* and *Enterococcus faecalis*. Samples from four dogs yielded *Mycoplasma* and a sample from one dog yielded *Ureaplasma* sp. In the 48 hour samples kept under refrigeration in Chill 5, there was no growth. Samples from seven dogs yielded *Mycoplasma* and samples from four dogs yielded *Ureaplasma* sp. In the 48 hour samples kept at room temperature in Chill 5, the sample from one dog had growth of *E. coli*. Samples from four dogs yielded *Mycoplasma* and samples from four dogs yielded *Ureaplasma* sp.

Percentage progressively motile spermatozoa in neat samples ranged from excellent to poor; with half of the dogs having acceptable motility (greater than 70%; Table 2). One dog was azoospermic.

The percentage progressively motile spermatozoa in the three dogs that were followed for the duration of the study declined during the first 24 hours. Change in percentage progressive motility was variable thereafter, with individual dog difference in the different extenders and at differing temperatures (Table 3). At the 48 hour mark, only 3 of the 12 samples would be deemed to have adequate motility (≥ 50%) in either the refrigerated or room temperature samples (Table 3).

Discussion

This study had several limitations. A larger sample size with greater breed diversity would have better reflected the population of dogs from which semen is collected and shipped in the United States. Adding a neat sample as an un-extended control to determine if the bacterial population changed over the 48 hour time period and the differences noted between refrigeration and room temperature also would have added value to the study. The authors chose not to add this sample because it does not mirror what happens in veterinary practice. A final limitation was lack of assessment of sperm motility in all samples immediately after collection and evaluation of samples from all dogs throughout the study. This would have better permitted the investigators to assess correlation between decreased motility and increased bacterial counts.

Thirty-five percent of dogs had significant growth of bacteria in their semen; this is in accord with information from the literature.[1] Bacterial growth was controlled in samples that were held at refrigerator temperature, but not in all samples that were held at room temperature. One dog had growth of bacteria that were not originally present; this may be because the antibiotic in the extender killed the bacteria in the neat sample and allowed organisms that were present in very small amounts to proliferate. The same can be seen in the Time 0 extended samples; anaerobic bacteria that were not seen in the neat sample were able to proliferate without the competition of other bacteria. This may potentially be a concern, as more pathogenic bacteria can survive in the face of antibiotics. It also makes one concerned for the possibility of increasing bacterial resistance. There was no discernible pattern to growth of *Mycoplasma* and *Ureaplasma* sp. in this study.

Extenders were not used in accordance with manufacturers' protocols, which recommend extension of the sperm-rich fraction only, at a dilution of 1:3 to 1:5. Again, the authors chose to mimic veterinary practice. Results may have been different if the samples had been extended as the manufacturer recommends, as the resulting extended sample would automatically have a lower concentration of bacteria due to the effect of dilution.

The authors note that reported percentage progressively motile spermatozoa in neat samples probably was not a true reflection of the dogs' semen quality, as samples were handled multiple times and were held at room temperature for two hours before evaluation. One dog was azoospermic. This could have been due to the fact that he was nervous for the collection or would have responded better if a teaser bitch had been present; this was not pursued as it was outside the purview of the study. The percentage of progressive sperm motility that was determined throughout the study showed that the sperm responded differently to the two extenders and that motility in most cases was adequate at the 24 hour mark. Because the entire ejaculate, not just the sperm-rich fraction, was used and manufacturers' protocols were not followed could explain why motility was less than that which may be seen in practice. The sperm did show some agglutination and it is not known what effect it would have on fertility.

Conclusion

Practicing veterinarians should use caution when using semen samples that may not have been handled appropriately or that arrive warm. If the sample arrives within the appropriate time and is still chilled, it appears that clinicians need not worry about excessive bacterial growth in those samples. Sperm motility was not affected by the presence of bacteria or *Mycoplasma* and *Ureaplasma* sp. therefore cannot be used to determine whether or not there are bacteria present in the sample.

Acknowledgements

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Figure: Schematic view of study

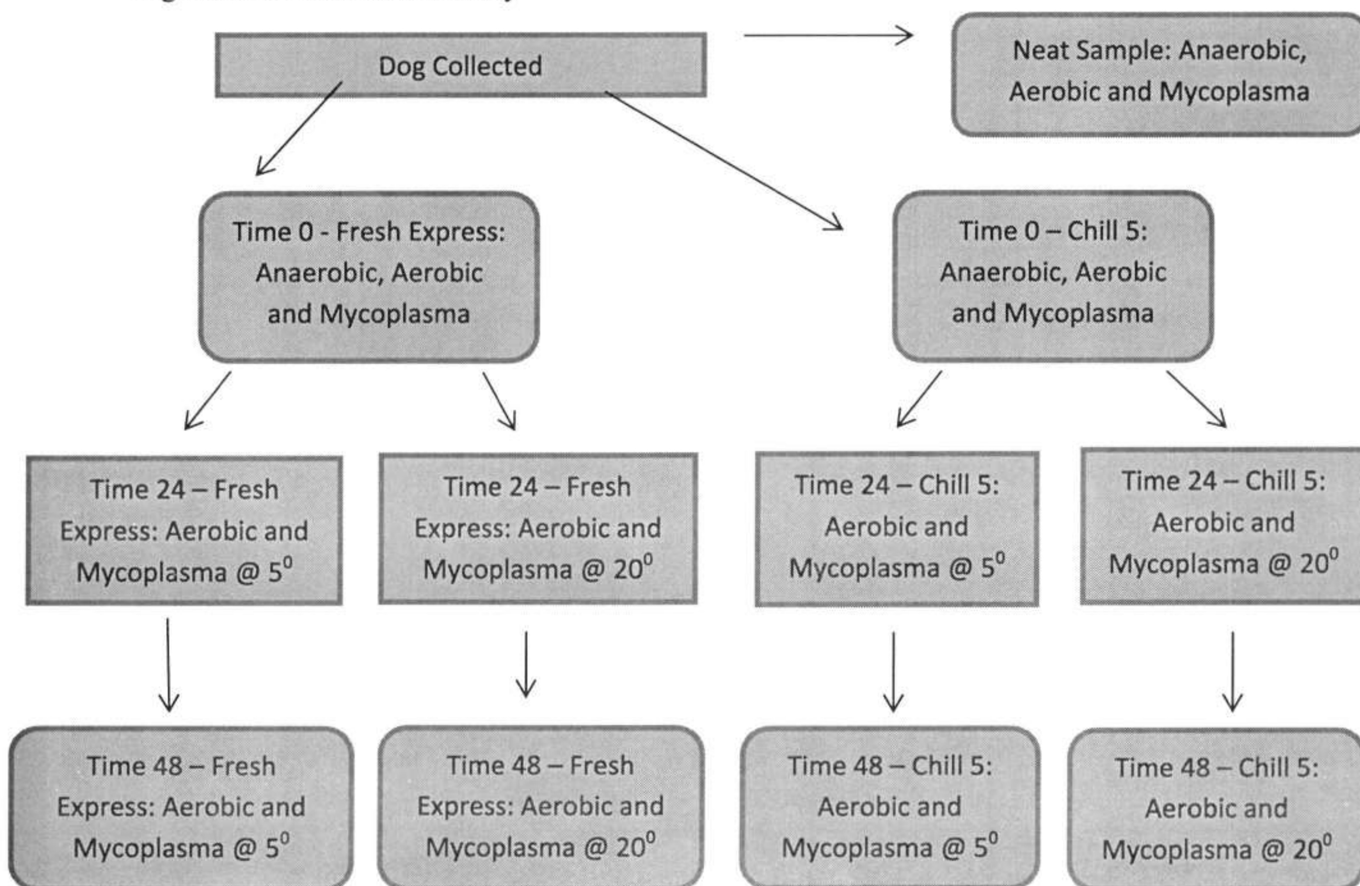

Table 1: Culture results of the dog's semen neat, in both extenders at Time 0, 24 hour and 48 hours at 5° and 20°C.

Animal Number	Neat Time 0	FE Time 0	Chill 5 Time 0	FE @ 5 Time 24	FE @ 20 Time 24	C5 @ 5 Time 24	C5 @ 20 Time 24	FE @ 5 Time 48	FE @ 20 Time 48	C5 @ 5 Time 48	C5 @ 20 Time 48
101	S1, L4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
105	S1	15	S3	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
106	S1, L2, M4	S5, L6	S6	Neg	Neg	15	15	Neg	Neg	Neg	Neg
107	N2, M3, S1	S2, S3	L2, M3	S2, S3	S2	L2, L3	S2, L3	S2	N2	S2, L3	N2
108	S1, L3	S3, S6	S3	Neg	Neg	S3	Neg	Neg	Neg	Neg	Neg
112	L1, 16	Neg	16	Neg	Neg	15, 16	16	16	Neg	Neg	16
113	L1, N2, M3, 15, 16	S1, L2, L7	S1, L2, L3, 15, 16	S1, L2	S1, L2	L2, L3, 15, 16	L2, L3, 15, 16	S2, 15, 16	L2, 15	S1, L2, L3, 15	S1, L2, 15
114	L1, M2, M3, 15	S1, L2, L7	S1, L2, L3, 15	S1, L2, 15, 16	L2, 15	L1, 15	S1, L2, 15	L2, 15	L2, 15	S1, L2, L3, 15	L2, 15
115	L1	L8, S9	L1, 15	S1	S8, M10	S8	L1, 15	S1, 15	N11, 15	S1, 15	S8, S12
116	L1, 15	S3, L6, S12, 15	S1, L3, 15	S3, S12, 15, 16	16	S1, S3, 15, 16	S1, 15, 16	15, 16	16	S1, L3, 15, 16	15, 16
117	M1, 15, 16	S1, L8 15, 16	S1, L8, 16	S8, 16	S1, 16	L8, 15, 16	L1, 16	L1, 15, 16	L1, 15	L1, 15, 16	L1, 16
118	L1, 16	S1, 16	S1, 16	S1, 16	S8, 16	S1, 15, 16	S1, 16	S8, S13, 16	S1	S1, 16	16
119	L1	Neg	Neg	Neg	Neg	Neg	15	15	Neg	15	Neg
120	L1, 15, 16	15	S14, 15	16	Neg	15, 16	15	16	Neg	15, 16	15

Key: S=Scant; L=Light; M=Moderate; N=Numerous; 1=Mixed Urogenital/Skin Flora; 2=*E. coli*; 3=Beta-hemolytic *Streptococcus* group G; 4=*Enterobacter aerogenes*; 5=*Bacteroides fragilis*; 6=gram negative bacillus (*Hemophilus* or *Pasteurella*); 7=*Hemophilus hemoglobinophilus*; 8=*Staphylococcus warnei*; 9=*Clostridium perfringens*; 10=*Enterococcus* sp.; 11=*Enterococcus faecalis*; 12=*Bacillus* sp.; 13=*Coryneform bacilli*; 14=*Propionibacterium acnes*; 15=*Mycoplasma*; 16=*Ureaplasma*

Table 2: Percentage progressively motile spermatozoa in neat samples

Animal Identification	Motility results
101	50%
105	20%
106	Azoospermic
107	10%
108	>90%
112	>90%
113	70%
114	70%
115	60%
116	>90%
117	60%
118	20%
119	80%
120	70%

Table 3: Percentage progressively motile spermatozoa from three dogs by extender, temperature, and time in storage

DOG	DOG 120		DOG 112		DOG 108	
NEAT	>90%		>90%		>90%	
	FE	C5	FE	C5	FE	C5
TIME 0	70	80	90	95	90	95
24 HRS 5°C	30	50	50*	35*	20	50
24 HRS 20-25°C	70	40*	10*	70*	55*	15*
48 HRS 5°C	25*	40*	0*	50*	0	0
48 HRS 20-25°C	50*	50*	5	10	10	0

*denotes sperm agglutination

Safety and efficacy of viral vaccines for bovine reproductive pathogens

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When considering safety and efficacy in the context of viral vaccines for bovine reproductive pathogens, safety is considered to be the lack of post-vaccinal adverse events for the vaccinated bull, heifer or cow and the associated semen, oocyte, zygote, gestating embryo, developing fetus, and newborn calf; efficacy is considered to be the resulting protection of health after post-vaccinal exposure to the pathogen of the vaccinated sire or dam and the associated semen, oocyte, zygote, gestating embryo, developing fetus, and newborn calf.

As bovine viral diarrhea virus (BVDV) and bovine herpes virus-1 (infectious bovine rhinotracheitis [IBR] virus; infectious pustular vulvovaginitis virus) are the most common viral pathogens impacting bovine reproduction in the United States, the safety of BVDV and IBR viral vaccines will be the primary focus of this manuscript. While some serotypes of bluetongue virus (BTV) do cause reproductive loss in cattle in the United States, the impact of currently circulating North American serotypes are frequently subclinical;[1,2] thus, the perceived need for immunization of cattle in the United States with BTV is currently very low. Modified-live vaccinal strains of BTV can clearly be acquired and transmitted by insect vectors.[1] Strains of BTV in modified-live vaccines also exhibit the capacity to cross the placenta and infect the bovine fetus resulting in a teratogenic outcome. Inactivated BTV vaccines approved for use in cattle exhibit little if any commercial availability in the United States. The relatively slow onset of immunity compared to modified-live vaccines and the necessity of serotype-specific vaccine formulations negatively impacts the potential commercialization of inactivated BTV vaccines based on the current ecology of this non-zoonotic pathogen in the United States.[1]

When considering the safety and efficacy of vaccines containing BVDV and IBR virus, the persistence of BVDV and the latency of IBR virus in some cattle may confound an overly simplified interpretation of safety and efficacy of vaccine use in the field. In an idealistic manner, we often postulate that cattle will not be exposed to a pathogen prior to vaccination and that a single dose of vaccine will result in consistent and almost instantaneous effective immunity ensuring health of the vaccinate and developing offspring despite later exposure to BVDV or IBR virus. Conversely, in a rather capricious manner, we often consider any indicator of disease within the 60 days immediately after vaccination to be due to lack of safety of available vaccines. This overly simplified mindset often leads to two tenets which may be erroneous: (a) lack of vaccinal safety is demonstrated if a viral pathogen is detected in association with any indicator of disease within the 60 days immediately after vaccination, and (b) lack of vaccinal efficacy is demonstrated if a viral pathogen is detected in association with any indicator of disease more than 60 days after vaccination.

Clearly, vaccination with modified-live or inactivated BVDV of cattle that are persistently infected does not consistently result in clearance of the persisting virus or clinical disease.[3] Thus, in contrast to the stated tenets, detection of BVDV associated with disease of vaccinates may indicate a persistent infection that was initiated prior to vaccination rather than lack of either vaccinal safety or efficacy. In comparison, vaccination with modified-live or inactivated IBR virus of cattle that are latently infected prior to vaccination will not result in clearance of the latent virus just as the animal's initial immune response was unable to result in clearance of the latent virus.[4] Thus, in contrast to the stated tenets, detection of IBR virus associated with disease of vaccinates may indicate initiation of a latent infection prior to vaccination rather than lack of either vaccinal safety or efficacy.

In addition to the confounding of tenets by enduring infections initiated prior to vaccination, mishandling of vaccines can result in confounding situations regarding vaccinal safety or efficacy.[5] Mishandling of vaccines may be categorized as (a) failure to administer the appropriate initial number of doses with the proper inter-dose time interval, (b) failure to administer vaccine to cattle of appropriate reproductive status as per label directions, (c) failure to observe label directions regarding the time interval between vaccine administration and attempted breeding, (d) failure to administer vaccine prior to the labelled expiration date, (e) maintaining vaccines at improper storage temperatures, and (f) failure to

observe labelled directions regarding the time interval between vaccine reconstitution and administration. Concerns about the shedding of BVDV and IBR virus to contacted herd mates after administration of modified-live vaccines appears to be overemphasized based on studies involving administration of Bovishield 4® and Express 5®.[6,7]

Clearly, issues that may occur in synchrony with vaccination for reproductive pathogens may cause health issues associated with BVDV and IBR virus. Comingling of diverse groups of cattle for vaccination prior to breeding can effectively result in transmission of BVDV and IBR virus.[8-10] Contamination of vials of vaccines lacking BVDV with nasal secretions from a persistently infected animal can result in transmission of BVDV.[11] Contamination of vaccines with virulent field strains of BVDV from fetal bovine serum can be associated with notable disease 12 to 13 days after vaccination;[12-14] conversely, contamination of a vaccine with a less virulent field strain of BVDV from fetal bovine serum serendipitously can be associated with unchanged safety and increased breadth of BVDV protection.[15]

Administration of modified-live vaccines containing BVDV and IBR virus is commonly recommended at least 30 days prior to breeding. In a small randomized, controlled clinical trial, pregnancy rates were reduced by 30% when a vaccine containing modified-live IBR virus was administered to seronegative heifers synchronous with the second dose of prostaglandin at the initiation of a 35-day breeding season.[16] While administration of vaccine to heifers shortly before breeding is commonly revaccination rather than the initial vaccination, producers are encouraged to avoid compression of this labelled interval. Though the goal of vaccination is immunization, even under optimal conditions a percentage of cattle may not seroconvert.[17] It would seem illogical to expect 100% of the vaccinated cattle to become immunized after the initial dose; thus, for some cattle the second dose of vaccine should precede breeding by the 30 day interval to achieve optimal reproductive results. This same principle explains why the administration of modified-live vaccine containing IBR virus during pregnancy after a single pre-breeding dose of the vaccine may lead to abortion in a small subset of animals. This outcome is demonstrated by the detection of IBR virus associated with one abortion in 235 cattle (0.4 %) revaccinated during the second trimester of gestation in a recent randomized, controlled trial.[18]

While the safety of modified-live vaccines containing BVDV and IBR virus is less than the safety of killed vaccines, the efficacy of modified-live vaccines containing these pathogens notably surpasses the efficacy of killed vaccines in providing fetal and abortive protection in research studies involving natural field exposures. For example, two doses of an inactivated BVDV vaccine administered 69 days prior to exposure with four persistently infected cattle for 98 days resulted in protection of 73% of the developing fetuses from BVDV[19] compared to two doses of a modified-live BVDV vaccine administered 102 days prior to exposure with three persistently infected cattle for 56 days resulted in protection of 100% of the developing fetuses from BVDV.[20] Thus, the selection of vaccine protocols for bovine reproductive pathogens requires a consideration of risk and risk tolerance. This balance may cause many producers to justify the administration to pre-breeding heifers of modified-live vaccines containing BVDV and IBR virus and the administration to pregnant cows of killed vaccines annually thereafter. This compromise may mitigate concerns about the safety of administration of modified-live vaccines during pregnancy and concerns about the efficacy of administration of killed vaccines without the initial activation of immunity associated with modified-live vaccines. While this compromise appears to be appealing, publications are not currently available of randomized, controlled trials describing the efficacy of such hybrid vaccination protocols. Demonstrated efficacy of such hybrid vaccination protocols that meets or exceeds the efficacy of pre-breeding vaccination with modified-live vaccines followed by revaccination during pregnancy with modified-live vaccines would clearly support the adoption or continued use of described protocols in the field.

In conclusion, the safety and efficacy of vaccines containing BVDV and IBR viruses must be carefully considered based on (a) the appreciation of disease risks in specific production-management systems, (b) an understanding of pathogenesis of bovine infections, (c) knowledge of the indications and hazards of available vaccines, and (d) the most appropriate timing of vaccination to achieve

immunization. Careful consideration of these factors may result in selection of optimal vaccination protocols for BVDV and IBR virus that maximize safety and efficacy to optimize bovine reproduction despite the potential for exposure to pathogens.

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Update on testicular infections with bovine viral diarrhea virus

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Bovine viral diarrhea virus (BVDV) is the prototypical member of the *Pestivirus* genus of the *Flaviviridae* family and is a significant cause of disease and economic losses in cattle herds worldwide. Clinical disease is manifested in several body systems including the respiratory, gastrointestinal, nervous and immune systems. However, the effects of infection on reproduction likely have the largest economic impact for the producer. In the female, the consequence of BVDV infection depends largely on the immune status of the dam and the stage of gestation at the time of infection. Conception rates may be negatively impacted if infection occurs near the time of breeding[1] and abortion can occur at any time during gestation. Infection of susceptible cattle with a noncytopathic strain of the virus between 18 and 125 days of gestation may result in immunotolerance in the fetus and persistent infection. Persistently infected (PI) animals serve as a viral reservoir and consistently shed high amounts of infectious virus throughout their lifetime. Infection of the pregnant dam may also result in several congenital defects, including cerebellar hypoplasia, hydranencephaly and microphthalmia if the event occurs during critical times of organogenesis.

Infection in the bull is not without consequence and can be classified into four distinct syndromes; transient infection, persistent infection, persistent testicular infection (PTI) and prolonged testicular infection. In addition to systemic clinical signs seen in cows and heifers, transient and persistent infection in bulls results in shedding of the virus through the semen. Viral shedding in the raw semen of transiently infected bulls has been detected at concentrations of 5 to 75 $CCID_{50}$/mL[2,3] and persists until the development of serum neutralizing antibodies (14 to 28 days post-infection). Concentrations of 100 to 200 $CCID_{50}$/mL have been detected in extended semen.[4] Viral shedding through semen in PI bulls occurs at much higher concentrations than in transient infections, with reported ranges of 10^4 to 10^7 $CCID_{50}$/mL in both raw and extended semen.[2,5,6] Virus shed in the semen is infectious and capable of being transmitted to susceptible animals.

Persistent testicular infection was first reported after a localized, persistent BVDV infection was documented in the testes of a seropositive, nonviremic bull at an aftificial insemination (AI) center.[3] The infection resulted in the continuous shedding of infectious BVDV in semen throughout the life of the bull despite the presence of neutralizing antibodies to BVDV in serum. The concentration of virus detected was intermediate between the level of viral shedding commonly seen in transiently (5 to 75 $CCID_{50}$/mL) and persistently (10^4 to 10^7 $CCID_{50}$/mL) infected bulls. When used to inseminate a seronegative heifer, semen from the bull resulted in infection of the heifer and subsequent seroconversion.[9] After euthanasia of the bull at the age of nearly 22 months, virus was isolated only from testicular tissue.

Localized testicular infections have been produced experimentally in post-pubertal seronegative, non-viremic bulls following intranasal inoculation[5] or subcutaneous vaccination[7] with a noncytopathic type 1a BVDV. Infections persisted for at least seven months in some bulls and infectious virus could be isolated from testicular tissue for over one year following challenge. Viral nucleic acid could be detected in semen by reverse-transcription nested PCR for at least 33 months post-inoculation. However, live virus was undetectable in semen, even when using the more sensitive roller bottle virus isolation technique. Challenge studies using semen from bulls with prolonged testicular infection failed to yield evidence of transmission when seronegative heifers were inseminated.[8] Consequently, this syndrome was termed prolonged testicular infection to differentiate it from other longstanding BVDV infections in cattle known to have significant epidemiologic consequences. In other words, the distinguishing characteristic of persistent and prolonged testicular infections is not the duration of infection but the presence or absence of infectious virus isolated from semen.

A second case of PTI was subsequently reported, also identified in a bull at an AI center.[10] Like the first bull, this second case was found to shed infectious virus in the semen while demonstrating no viremia. Viral concentration in processed straws of semen obtained from the bull between the ages of 16 and 22 months were similar to concentrations (10^2 to 10^3 $CCID_{50}$/mL) seen in semen from the first bull.

However, the detected concentration of BVDV was lower when semen samples were transported on ice as opposed to in liquid nitrogen.[10] Live virus was shed in the semen despite consistent serum antibody titers to BVDV1 $\geq$ 1:256 from the age of eight months onward, illustrating the immune privileged status of the testicles. Immunohistochemistry (IHC) staining of testicular tissue obtained by testicular biopsy at the age of 33 months confirmed the presence of BVDV within the tissue in association with Sertoli and germinal cells.

What is perhaps most notable about the second bull with PTI is that the bull was apparently able to clear the infection. Semen collected from the bull between the ages of 36 to 41 months was PCR positive for the presence of BVDV.[10] Routine isolation techniques failed to detect live virus from any samples obtained during this period although one sample collected from the bull at 36 months of age was virus isolation positive using the more sensitive roller bottle technique. All subsequent semen samples obtained from the age of 41 months until the bull was euthanized at 48 months of age were virus isolation and PCR negative. Additionally, IHC staining of testicular tissue following euthanasia failed to detect viral antigen, in contrast to the clear association of virus with Sertoli and germinal cells in testicular tissue 16 months earlier.

The apparent clearance of a PTI by this bull is surprising based on our current understanding of persistent BVDV infections. One obvious difference between persistent infection and PTI is the production of serum neutralizing antibodies to the infecting strain of the virus. Persistently infected animals are capable of mounting a serologic response to heterologous strains of the virus or strains closely related to the infecting strain but not to the infecting strain itself.[11,12] Both bulls demonstrating PTI exhibited a serum neutralizing antibody titer to the infecting strain causing the testicular infection.[3,10] However, the blood-testis barrier (BTB), formed between Sertoli cells of the seminiferous tubules at the time of puberty prevents the entry of serum antibodies to the site of spermatogenesis although trauma may allow the barrier to be breached. In this case, there was no known trauma to the testicles and the tissue was normal on histological examination. A testicular biopsy was performed on only one testicle; yet, at the time of the procedure, infectious virus could no longer be detected from semen using routine isolation techniques. Thus, the mechanism by which the bull resolved the testicular infection is unclear. Consequently, extended isolation with routine monitoring for the presence of infectious virus in the semen could be considered for valuable animals diagnosed with PTI in the future.

The pathophysiology of PTI in bulls is unknown and remains a topic of speculation. The BTB only becomes functional at the onset of puberty. Therefore, it has been speculated that a bull could become infected just prior to closure of the BTB, allowing entry of the virus into the immune privileged site but blocking the subsequent entry of neutralizing antibodies.[3] Transient BVDV infections in post-pubertal bulls can result in the isolation of virus from semen for < 21 days after infection[5] but a consistent shedding of infectious virus over a sustained period (as with PTI) is not seen. The length of viral shedding in semen following transient infection appears to be inversely related to the rapidity and magnitude of the host immune response.[5] However, both bulls with PTI maintained high neutralizing antibody titers to the infecting strain and yet shed high levels of virus in the semen.

It is unknown if the fact that both recorded cases of PTI have occurred in dairy bulls is a reflection of management factors important to the pathogenesis of infection, a reflection of sampling bias or random variation. Colostrum management of dairy bull calves is often less of a concern than that of dairy heifers. Thus, it is possible that a dairy bull calf with low levels of passive immunity is infected with the virus through exposure in milk from a PI animal[13] or through the respiratory tract. If the virus were able to elude the host immune response until the time of puberty it could be protected in the testes after formation of the BTB.

In summary, though much has been learned about testicular infections with BVDV, questions remain. Development and pathogenesis of testicular infections with BVDV remain to be clearly understood. Though testicular BVDV infections are believed to occur relatively infrequently, the incidence is likely underreported due to lack of testing or reliance on testing for the detection of PI animals that will not detect persistent or prolonged testicular infections (e.g., ear notch IHC, serial virus isolation). Semen containing infectious virus is capable of viral transmission to susceptible animals and

thus, out of caution, any semen testing positive for BVDV, regardless of testing method, should not be used for artificial insemination. Isolation and repeated testing of valuable animals diagnosed with PTI may be warranted given the reported clearance of PTI in a single bull.

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Epizootic bovine abortion (foothill abortion): the disease, diagnosis and control strategies

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Historical perspective

Epizootic bovine abortion (EBA) was recognized as a disease entity of beef cattle in the 1950's in California. The disease, referred to regionaly as foothill abortion, is believed to have existed in California as early as the 1920's. The name, foothill abortion, was appropriately coined as the disease was noted to occur exclusively in pregnant cattle that were grazed in foothill and mountainous regions in California. The economic impact of foothill abortion was of such significance that it contributed to the establishment of the University of California's School of Veterinary Medicine in Davis and research funds to study it were established as a permanent line-item in the state's legislative budget.

Keywords: Foothill abortion, epizootic bovine abortion, *Pajaroellobacter abortibovis*, *Ornithodoros coriaceus* , arthropod-borne disease

The disease

Foothill abortion is now recognized to occur in California, Southern Oregon and Nevada; occurrence in Southern Idaho is suspected, but not confirmed. The disease is expressed as a term abortion in beef cattle with gross and microscopic pathology sufficiently unique that it is typically diagnostic. A "foothill fetus" can present grossly in many different forms; gestational age at the time of infection and the associated fetal immune response contribute to the variation. The most typical presentation is that of a near-term haired-fetus with a pot-bellied appearance due to excessive accumulation of ascites, mucosal petechial hemorrhages in the eyelids, tongue and oral cavity, and enlarged, easily palpable prescapular lymph nodes. Occasionally, small denuded raised skin lesions may be seen. Fetuses can be aborted dead or delivered live, the latter typically dying within hours. A subset of infected calves born alive may survive for variable periods and would be considered weak calves. A smaller number of aborted affected fetuses will present without hair, with or without the pot-belly, and resemble the appearance of a pig. These latter fetuses are typically of a younger age and have undergone substantial autolysis. Upon revealing the abdominal cavity, via incision through the right side of the fetus, a variety of gross anomalies can be noted including excessive ascites and fibrin deposition, an enlarged mottled liver with rounded edges; the spleen can be sufficiently enlarged such that it is visible without manipulating the intestines. The mesenteric lymph nodes are typically enlarged and numerous, resembling grape clusters running the length of the small intestine. Extensive and enlarged visible lymph nodes are often present throughout the tissues. Grossly, the thymus presents with either distinct petechial hemorrhages on the white background or as a darkened tissue mass with extensive hemorrhage and necrosis and firm texture due to fibrin deposition. Microscopic examination of necropsy tissues reveals an extensive and acute vasculitis, focal-necrotizing lesions often resembling pyogranulomas in lymph nodes and spleen and a thymus characterized by atrophy of cortical thymocytes and infiltration by macrophages; these thymic lesions are pathognomonic for foothill abortion.

As a final note on the disease, recent studies testing the safety and efficacy of a live-virulent vaccine (see "Control" section below) have identified new and unexpected characteristics associated with infection by the causative bacterial pathogen, *Pajaroellobacter abortibovis*. Experimental infection of cattle within 3-4 weeks of breeding can result in embryonic mortalities approaching 10%. Affected animals can successfully breed-back if the bulls are maintained in the herd over the summer months but short breeding periods of 30 to 45 days can result in increased numbers of non-pregnant animals. The basis of this anomaly is assumed to be due to recent evidence suggesting the bacterial pathogen can remain active in an infected animal for upwards of 60 days post-infection.

Foothill abortion has never been described in any species other than the bovine. Attempts to experimentally infect pregnant sheep, resulting in abortion or birth of weak lambs, were unsuccessful.

The slow intracellular replication of the causative agent (~1 day) requires that a long gestational period be available for it to become disseminated throughout the body prior to development of a vigorous fetal immune response that ultimately contributes to lesion development and death. The gestational period in sheep, goats and deer is sufficiently short that the complex bacterial pathogenesis cannot unfold prior to birth.

The etiologic agent

The search for the etiologic agent of foothill abortion spanned 50 years and began in the 1950's with a focus on chlamydia and development of an associated vaccine that never caught traction. Upon identification that a unique tick, *Ornithodoros coriaceus,* (see "Disease transmission" section below) was the vector of foothill abortion, efforts were reinitiated to identify the causative agent, resulting in a focus on incriminating a retrovirus. These efforts were replaced in the 1980's with a focus on *Borrelia coriaciae* and unidentified spirochetes as potential causes of foothill abortion. All of these studies were hampered by an inability to experimentally transmit the disease with any consistency; feeding 100's of field-trapped *O. coriaceus* ticks on susceptible pregnant heifers was the only known way to transmit the disease and this was only successful approximately 50 percent of the time. Development of a reliable mechanism for transmission of foothill abortion in the 1990's using cryopreserved thymus from select aborted fetuses facilitated studies that ultimately demonstrated the causative agent to be antibiotic susceptible. This advance, associated with the application of modern molecular biology techniques, culminated in 2005 with identification of a unique bacterium within the Myxococcales order, as being the etiologic agent. This unique bacteria has tentatively been coined *Pajaroellobacter abortibovis*, based upon a combination of the name of the tick vector, type of microbe, the disease and the susceptible host.

Disease transmission

Foothill abortion is an arthropod-borne bacterial disease that is transmitted by *O. coriaceus*; commonly referred to as the Pajaroello tick, initially coined by native American Indians residing in the coastal regions of Central California. The tick was feared more than rattlesnakes due to a severe hypersensitivity that could be established following a first bite. The unique distribution of the Pajaroello tick in foothill and mountainous regions of California contributed to its' identification as being the vector of foothill abortion; the geographic distribution of the tick closely resembled the distribution of foothill abortion. While the geographic distribution of the tick extends down through Mexico and into Central America, the disease has never been reported south of the U.S border. The tick lives in the duff under trees and/or brush in arid environments, typically in mammalian bedding areas where they have somewhat reliable access to an occasional blood meal. The tick is attracted to CO_2 emitted by a warm-blooded host. Nymphs and adults obtain a blood meal within 20 to 30 minutes, then detach and fall back into the duff; larvae can remain on the host for up to a week and are believed to be responsible for slowly extending the distribution of the tick and the disease in an eastward direction. The tick undergoes multiple stages of development from the hatched larvae through nymph stages, ultimately becoming an adult. The tick appears to have the capacity to live in excess of ten years and larger ticks can survive for several years without a blood meal. Development of molecular diagnostic probes (see "Diagnosis" section) has permitted researchers to identify the presence of *P. abortibovis* in the salivary gland of field-collected ticks. Approximately 10 to 20% of ticks are infected, but only a small percentage (probably in the range of 1-2%) harbor sufficient bacteria to transmit the disease. No associations have been identified between presence of the bacteria with tick age, size or sex; transovarial transmission of the bacteria in ticks has not been demonstrated. The reservoir of the bacterial pathogen is currently unknown and could include the soil/duff in which the ticks live and/or unidentified mammalian and avian hosts.

Diagnosis

Diagnosis of foothill abortion has classically been based upon a combination of gross and microscopic anatomy, elevated levels of fetal immunoglobulin (Ig) and knowledge of dam exposure to areas inhabited by the Pajaroello tick vector during the window in which the fetus is susceptible to

infection (~60-150 days gestation). While a "classical" presentation of foothill abortiom lends itself to a definitive diagnosis based upon pathognomonic lesions in the thymus, not all cases are straightforward. An immunohistochemistry assay is now routinely used as an adjunct to diagnosis by facilitating microscopic visualization of the causative bacteria in thin sections derived from formalin fixed necropsy tissues. A molecular diagnostic, based upon a polymerase chain reaction (PCR) applied to fetal necropsy tissues, has also proven to be a valuable tool in supporting diagnosis of foothill abortion, especially when the fetus has been badly scavenged by predators. A serologic assay for identification of *P. abortibovis*-specific antibodies in fetal serum or other body fluid has successfully been developed. This serologic assay is proving to be useful in identifying *P. abortibovis*-associated abortions, including aborted fetuses and weak calves that have largely cleared the bacterial infection. Serologic diagnosis requires that the subject has not ingested colostrum. The identification of antibodies specific for *P. abortibovis* have recently been identified in a few term abortions and apparent dystocias that did not present as foothill abortiom based upon gross and microscopic pathology. The significance of such infections on near-term losses is currently unknown. Lastly, the serologic assay is being applied to identify past exposure of mature cattle to *P. abortibovis* to better define the geographic distribution of the pathogen in a manner that is not dependent upon collection of the Pajaroello tick or diagnosis of the disease.

Control

Control of foothill abortion has classically been limited to alteration of management practices that can sometimes reduce, though not eliminate, foothill abortion-associated losses. Two often-used techniques include: i) minimizing exposure of naïve replacement heifers to tick habitat during the gestational period in which the fetus is susceptible (currently considered to be about 60 to 150 days gestation) or ii) attempting to expose naive replacements to the tick vector prior to breeding. Some producers have reported success in controlling foothill abortion by running feeder steers on a tick habitat in the spring in an attempt to "feed up" the ticks prior to introduction of susceptible pregnant heifers into that same habitat. The logic behind this approach is to reduce the number of hungry ticks in the area for several months, allowing the heifer's developing fetus to sufficiently mature beyond 150 days gestation. A well-established approach to managing foothill abortion is breeding in the winter months when the ticks are at minimal activity and calving in the fall. This is more practical in moderate climates with winter grass range such as California's coastal range and low-elevation foothill regions of the Sierra Nevada mountains bordering the Central Valley. The greatest losses to foothill abortion typically occur when naïve pregnant cattle are introduced from non-endemic areas into Pajaroello tick habitat. Such practices should be avoided at all costs as losses can approach 90%.

Development of a vaccine for foothill abortion became a priority upon identification of the causative bacterial agent of foothill abortion, *P. abortibovis*. Efforts to propagate the bacteria in a variety of synthetic media and primary and secondary cell culture systems were met with limited success. The successful infection of mice with severe-combined immunodeficiency (SCID) provided a potential way forward for vaccine production. Infection of SCID mice with *P. abortibovis*, derived from fetal bovine necropsy tissues, results in development of a wasting disease at 60-70 days post-inoculation. While lesions typical of foothill abortion are absent in necropsied mice, spleens are enlarged and contain viable intracellular bacteria. Spleens can be converted into single-cell suspensions by pressing the macerated organ through sterile screen meshes; such cells can be successfully rate-frozen and cryopreserved in liquid nitrogen with long-term maintenance of cell and bacterial viability. A flow-cytometric technique has been developed to determine the percentage of splenocytes harboring bacteria. The ability to quantitate the number of infected cells in a given population of murine splenocytes provided the necessary quality control (ability to control the dose of bacteria) to proceed with development of an experimental live virulent vaccine for foothill abortion. Naïve heifers that were vaccinated, then bred and challenged at the peak of fetal susceptibility (90-100 days gestation) produced healthy calves while the majority of negative controls had term abortions. Preliminary field studies with the experimental foothill vaccine have been conducted in multiple herds in California and Nevada with no systemic reactions, minor reactions at the vaccine injection site and no documented cases of foothill abortion. To date, the

vaccine has enjoyed 100% success in preventing foothill abortion under both experimental and field conditions. Efforts are currently underway at the University of California-Davis to license and commercialize the cryopreserved vaccine.

Economic impact

Foothill abortion has, and continues to be, the #1 disease in California negatively impacting calf production by beef producers; the relative impact in Oregon and Nevada is unknown. The economic impact of "foothill" is exaggerated due to the heifer/cow's production being lost for an entire year since the abortion occurs at term. Additional impacts of the term abortion, typically in replacement heifers, are associated with impaired clearance of the placenta that is often associated with compromised subsequent conception and irreparable damage to the distended udder in animals grazed in brushy country. Altering management practices to reduce foothill abortion losses in endemic areas by fall-calving (discussed in the "Control" section) can result in reduced feed efficiency on summer range, especially on ranches east of the Sierra Nevada and Cascade Mountains where cattle must be fed hay over the winter months.

Chlamydia and Chlamydophilia in bovine reproduction

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Abstract
Infection with chlamydial bacteria, specifically *Chlamydophila pecorum*, *C. abortus*, and *C. psittaci*, is common in cattle. Infection can cause different diseases of which sub- and infertility are the most often observed clinical manifestations. While bulls are usually carriers of chlamydia bearing the risk of being vectors for an infection of females, either through natural mating or artificial insemination, heifers and cows can suffer a multitude of genital disorders. However, chlamydiosis in cattle is generally considered a (multi)factorial disease with factors being related to the environment, confinement or climate. This contribution will briefly describe chlamydial infection in cattle with specific focus on reproduction.

Keywords: Chlamydia, Chlamydophilia, bovine, reproduction

Introduction

Chlamydiae are obligate intra-cellular gram-negative bacteria that cause different diseases in animals and humans.[1] In cattle, chlamydial infections can cause abortion,[2] polyarthritis,[3,4] encephalomyelitis,[5] keratoconjunctivitis,[6] pneumonia,[7] enteritis,[8] hepatitis,[9] vaginitis and endometritis,[10,11] infertility,[12,13] and chronic mastitis.[14] Besides clinical disease, Chlamydiae may be the cause of sub-clinical manifestation.[9,15-21] This is supported by observations in dairy herds with endemic infection and sub-optimal production records where other relevant pathogens were absent. Moreover, these observations have suggested a role for chlamydial infection as a multi-factorial disease complex.[22] This contribution will briefly review the current knowledge on chlamydial infection in cattle with emphasis on clinical reproduction.

Taxonomy

The order "Chlamydiales" has been re-classified regularly leading to the current taxonomy of the family Chlamydiaceae separated into the genera Chlamydophila and Chlamydia, with a total of nine species, namely, *Chlamydophila abortus*, *C. pecorum*, *C. psittaci*, *C. pneumoniae*, *C. felis*, *C. caviae*, *Chlamydia trachomatis*, *Chlamydia suis* and *Chlamydia muridarum*. Three new families were added, i.e. Parachlamydiaceae, Waddliaceae, and Simkaniaceae.[23]

There are a number of chlamydial species that can be observed in cattle including *C. pecorum*, *C. abortus*, *C. psittaci*, and *Chlamydia suis*, with the first three being clearly the most prevalent species.[2,13,21,22,24-27,30-32]

Prevalence

Chlamydial infection is highly prevalent in the dairy cattle population worldwide, with seropositivity at herd level ranging from 45% to 100%. This suggests that chlamydial infections are ubiquitous in cattle.[33] Generally, serological data are difficult to interpret, as chlamydial antibodies can be the humoral response from an infection in any part of the body, and a bacteriologically positive result not necessarily means that the individual has developed antibodies.[32] For instance, in a recent survey conducted in six bull studs it was demonstrated that, while seropositivity was 51% the rate of PCR positivity in semen, preputial washes and feces was between 9% and 18%. Furthermore, serology failed to identify bulls shedding chlamydiae in their semen.[28] Similarly, in a study aimed to monitor the course of a "natural chlamydial infection" in calves it was found that 60% of the animals seroconverted after inoculation, whereas all were shedding the bacteria.[20]

As aforementioned, *C. pecorum*, *C. abortus* and *C. psittaci* are the most prevalent species in cattle. However, there seem to be specimen, gender and topographical effects. For instance, the study of

Kemmerling et al investigated vaginal swabs and revealed 56% positive for *C. psittaci*, 37% positive for *C. abortus* and 8% positive for *C. pecorum*.[21] In contrast, stud bulls were mostly *C. psittaci* positive in semen and preputial washings, while *C. pecorum* prevailed in fecal samples.[28] In Asia, specifically Taiwan, it seems that *C. abortus* is the most prevalent genital chlamydial species.[27] In the United States, previous studies have mostly demonstrated *C. pecorum* and *C. abortus*, while *C. psittaci* was not found.[17,19] But even within Europe, i.e. Austria and Switzerland, the most prevalent chlamydial species, respectively, were also found to be different.[2,30,33,34] Generally, the different chlamydial species found in cattle can infect different organs not uncommonly at the same time. On the other hand there seems to be a genital tropism, with *C. abortus* and *C. psittaci* being the ones that are mostly infecting the genital tract.[21,28,35] Mixed infections with two or more chlamydial species at the same time are always possible.[22]

Routes of transmission

Chlamydiae can be shed by almost all secretions and excretions (vaginal, ocular, and nasal fluids, semen, urine etc.), with fecal shedding being the most important route. Also, the bacteria can be found in aborted material such as fetuses as well as placentae.[20,21,28,36,37] Animals usually infect themselves via the horizontal route through ingestion or inhalation. Venereal transmission seems possible.[38,39] However, available data still raise questions for example of the bacterial load in semen that is necessary for an infection, the pathogenicity of different species, and possibly also the survivability of Chlamydiae after extension of semen during semen processing. Vertical transmission has yet not been reported.

Effects of chlamydial infection on health and fertility

Generally, infection with chlamydia is considered a multifactorial disease, with several factors related to housing, climate, hygiene etc. being contributors. Until recently, chlamydial infection was considered a sporadic event in cattle with no clinical signs or a sub-clinical course that does not have effects on the animal soundness. In a more recent study, however, where 100 randomly-selected dairy farms were investigated for the effects of chlamydial infection on production traits, a clear relationship was found to average annual milk production (8681 vs. 9197 kg in infected vs. non-infected animals), number of lactations/animal (2.4 vs. 2.9), rates of abortion, premature calving and perinatal calf deaths ($P<0.001$).[21] Most importantly, the study revealed that sanitation was better on Chlamydia negative farms again supporting the fact of a multifactorial genesis of Chlamydia-associated disease and that management factors can have a positive effect on the occurrence of the disease.[13]

Besides general health, Chlamydiae, specifically *C. abortus* and *C. pecorum* have been shown to effect milk yield, either by inflammation or subclinical disease.[14]

An infection with chlamydia may cause sub- or infertility. Infected bulls may suffer from vesiculitis,[40] or may not be affected at all.[28,29] Interestingly, infected bulls did not show impaired semen quality as measured on the basis of standard semen parameters.[28] As aforementioned, regardless of being diseased, bulls can shed Chlamydia via semen, and shedding can be intermitted which then puts some difficulties on diagnostics and interpretation of diagnostic results. An interesting finding by an in vitro study conducted by our group is that Chlamydia bind to specific receptors of the sperm membrane (spermadhesines) that are necessary for the sperm to be bound to oviductal cells of the sperm reservoir, i.e. the utero-tubal junction. While this may lead to a reduced capability of sperm to be attached to oviductal cells (and may thus impair the fertility and survivability of the sperm), this mechanism may also be part of an "active" transmission process of Chlamydia through semen.[41]

Any part of the genital tract of a cow or heifer can be infected. Swabs taken from different parts of the genital tract including vagina and uterus[30] but also the oviduct (Kauffold et al., unpublished) frequently proved positive for Chlamydia. Sporadic abortion can occur.[2,26,34] Also, sporadic cases of endometritis and vaginitis due to chlamydial infection has been described.[11,17,30] Interestingly, the fact that Chlamydia was found in different oviductal segments suggests that, as in humans, laboratory animals but also swine,[42] the oviduct is a target organ for an infection. Whether or not this may have consequences for oviductal functions is yet not known. However, the fact of an "oviductal postitivity" *per se* suggests that Chlamydia may damage or interfere with oviductal functions either structurally

and/or functionally. An oviductal infection may be the "ideal" explanation of subfertility or rebreeding of cows and heifers, that otherwise do not show any clinical disease.

Conclusions

There is clear evidence that chlamydiae are endemic in the cattle population. It is also clear that bacteria can cause clinical disease. However, subclinical cases are the predominant manifestation. Sub- and infertility are the most often seen clinical signs. Any part of the genital tract can be infected. Whether or not an infection results in structural and/or functional damage is dependent on a multitude of factors related to e.g. environment, confinement or climate. Thus, management measures that are aimed to improve these factors are beneficial toward avoiding chlamydiosis in cattle.

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Chlamydia and Chlamydophilia in small ruminants and other farm animals

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Abstract

Infection with *Chlamydiae*, specifically *Chlamydophila (Cp.) abortus*, causes enzootic abortion in small ruminants. The disease is highly prevalent in most parts of the world and has a high economic impact especially on the sheep industry. Swine is another species that can suffer from chlamydial infection. Besides *Cp. abortus*, *Cp. pecorum* and *Chlamydia (C.) suis* can all cause reproductive failure. Unlike in small ruminants, chlamydiosis usually occurs as a sporadic event. In swine, the disease has several clinical manifestations (e.g. abortion, endometritis, vaginitis, vulval discharge) and is considered a multifactorial disease. In both small ruminants and swine, males can also get infected and then become diseased, and may shed *Chlamydiae* with their semen. This contribution will briefly describe chlamydial infection in small ruminants and swine with specific focus on reproduction.

Keywords: *Chlamydia*, *Chlamydophilia*, small ruminants, swine, reproduction

Introduction

Chlamydiae are obligate intra-cellular gram-negative bacteria that cause different diseases in animals and humans.[1] The bacteria is widespread in the cattle population were it can infect different organs or tissues and can cause several albeit mostly sporadic diseases including reproductive failure.[2] This is similar to the situation seen in swine where intestinal and pulmonary but also genital infection can occur.[3] In contrast, an infection in small ruminants usually leads to an enzootic disease with the main clinical sign of abortion.[4] This contribution will briefly review the current knowledge on chlamydial infection in small ruminants and swine as the most susceptible large animal species, in addition to cattle, to chlamydial infection. Special emphasis will be put on clinical reproduction.

Chlamydia and Chlamydophila in small ruminants

Prevalence and species

Chlamydial infection is prevalent in the sheep and goat population worldwide, except in Australia and New Zealand.[4] The serological prevalence can vary in different countries and also between regions within a country. For instance, in a study conducted in Switzerland the overall flock sero-prevalence against *Cp. abortus* was 18.47 % (118/639), ranging 4.4-41.0% for different regions within the country.[5] In another study also conducted in Switzerland, sera from 775 randomly selected flocks out of 11 cantons were investigated and antibodies against *C abortus* found in almost 19% (144) of the 775 examined sheep flocks. The predominant species in small ruminants is *Cp. abortus*, while *Cp. pecorum* can also be observed.[6] In a study conducted in the Netherlands during five successive lambing seasons between 2006 and 2011, 453 submissions of abortion material, 282 of ovine and 171 of caprine origin, were examined at the Animal Health Service in the Netherlands. Infectious agents as the most plausible cause of the abortion were found in 48 percent of the ovine submissions and in 34 percent of the caprine submissions.[7] Similarly, in another study coming from Switzerland where the causative situation of sheep and goat abortions was investigated, *Cp. abortus* was found in 39% of sheep and 23% of goat abortions.[8]

Rams and bucks were also found serologically positive indicating previous Chlamydia exposure. In a Swiss study 34.8% of the rams (16/46) and 60% of the bucks (9/15) from different flocks were serologically tested positive for *Cp. abortus*.[9] However, none of the rams or bucks had a Chlamydia positive semen result. In contrast, when rams were experimentally infected with *Cp. abortus*, they developed clinical disease and shed *Chlamydiae* with their semen.[10,11]

Transmission

The infection occurs mostly orally with contaminated environmental material such as bedding material and feed, as well as with contaminated fomites.[10-12] Diseased animals (i.e. after abortion; see below) will shed the bacteria through vaginal secretions, placentas and (dead or weak) fetuses.[10,13] Lambs born to infected animals may also carry *Chlamaydiae* and then serve as a vector. It is interesting to note that the viability of *Cp. abortus* in the environment can be variable, with weeks to months viability in dry straw or "dried up" placentas at cold ambient temperatures (during the winter months).[10,12,14] The possibility of venereal transmission remains unclear.

Genital infection and clinical disease

Cp. abortus causes enzootic abortion is recognized as a major cause of reproductive loss in sheep and goats worldwide. In countries of Northern Europe, the disease is the most common infectious cause of abortion in small ruminants, accounting for approximately 20 to 45 % of all abortions (based on data from Switzerland, Netherlands and UK).[4,7,8] Stuen and Longbottom[4] recently published a review in which they comprehensively described *Cp. abortus*-induced disease. Accordingly, an infection in animals is asymptomatic, displaying no specific premonitory signs of the impending abortion. Only some behavioral changes or a slight vaginal discharge may be observed in a few animals up to a couple of days before abortion.[15,16] Regardless of when the infection occurs, the bacteria starts to invade the placenta at 90 days' gestation and then causes suppurative necrotizing placentitis. The first clinical sign of the endemic disease usually starts with the first delivery of dead lambs two to three weeks before the expected lambing. Aborted lambs appear normal and well-developed, but some may show a degree of edema giving rise to a "pot-bellied" appearance. The fleece may be discolored or covered with a pinkish-brown material that is usually a sign of delayed parturition and originates from meconium. The placental membranes can be necrotic.[15,16] An infectious vaginal discharge may be observed for several days following abortion, but otherwise the animals are clinically normal and are considered immune to further disease lifelong. Retained placenta can be occasionally observed and is more frequent in goats than in sheep.[17,18] This can result in the development of metritis and eventually, death due to secondary bacterial infections. In addition to abortion, animals may deliver stillborn or weak lambs that fail to survive beyond two days of age. Also, it is not uncommon for infected animals to deliver healthy lambs, with little necrotic damage evident in the placental membranes, as well as delivering one dead and one weak or healthy lamb.

There are not a lot of data available with respect to clinical disease in rams in bucks. Experimental infection with *Cp. abortus* has resulted in epididymitis in rams.[19]

In contrast to *Cp. abortus*, *Cp. pecorum* causes subclinical intestinal disease, but has however, also been reported to be involved in the conjunctivitis-arthritis complex, and may also cause pneumonia.[20,21]

Chlamydia and Chlamydophila in swine

Prevalence and species

Chlamydiae are widespread in female pig breeding stock worldwide. Serological testing revealed a prevalence of chlamydial infections ranging between 16.4% and 63.5%.[22,23] Seroprevalence surveys conducted in several European countries indicated, however, different antigen pressure at different locations, even within the same country.[23,24] Clearly, overall prevalence data only partially reflect genital tract infection, as many other organs besides the genital tract including intestines, lung, udder or joints can be colonized,[25-28] and thus be the primary site of antibody response.[29,30] Chlamydial DNA has been frequently detected in several female genital tract specimens, such as from cervical swabs, uterine and oviductal tissues, and from aborted material. However, the rate of positivity varied considerably among studies and according to the examined specimens, ranged between 3.9% from fetal aborted membranes[31] to 61.9% from uteri and oviducts of cull pigs.[32] Chlamydia has also been detected in uteri of feral swine.[33]

There has not been as much testing on boars as on sows for the prevalence of *Chlamydiae*. However, seropositivity rate was equally high,[27,30] and antigen pressure seems to vary also for boars, as seropositivity rates differed between studs.[30] Raw semen samples were detected positive for *Chlamydiae*, however, the percentages of positive samples varied notably between 9.1 and 17.2%.[34,35] Moreover, shedding in individual boars seems to occur inconsistently.[35] Studies on *Chlamydiae* in the boar's genitourinary tract yielded ambiguous results. Tenakum et al for instance, were unable to find *Chlamydiae* in testicles, epididymides, accessory glands, prepuce and urethra of 41 sexually active or virgin Swiss boars using 16S rRNA-PCR and immunohistochemistry.[35] If, however, genitally diseased boars were investigated, *Chlamydiae* could be detected within the genitourinary tract by either culture or PCR.[36,37]

Both *C. suis* (formerly *C. trachomatis*) and *Cp. abortus* were most frequently observed in genital tract tissues of female pigs. While *C. psittaci* and *Cp. abortus* predominated in cervical swabs[38,39] and/or genital tract tissues,[29,40] *C. trachomatis* was more frequently found in aborted material.[41] A third species, *Cp. pecorum*, was retrospectively identified from cases of abortion.[25,42] Moreover, *Cp. psittaci* (the avian strain of *C. psittaci*) has been detected in uteri and/or oviducts of cull sows[32,40] and feral swine.[33] Dual infections, such as with *C. trachomatis*/ *Cp. pecorum*, *Cp. abortus*/*C. suis* and *Cp. psittaci*/*C. suis* are possible. Recently, *C. trachomatis* (a human strain) has been described in three specimens of the female genital tract;[32] the relevance of this finding is, however, unclear. Besides *Chlamydiae*, Chlamydia-like organisms have been detected in cervical swabs, uteri, oviducts and aborted

material.[29] Boar semen was tested positive for *C. suis*, *Cp. psittaci* and *Cp. pecorum*, but not for *Cp. abortus*.[30,35] Chlamydia-like organisms were also found in semen,[35] as well as dual infections observed.[30]

Transmission

Transmission and genital infection has not been very intensively investigated in swine, and data are thus patchy and inconclusive. In general, infection in swine is assumed to occur oro-nasally after exposure to, or consumption of infected environmental material,[43,44] but infection by the aerosol route is also possible.[28,45] Chlamydia might then be systemically disseminated into the genital tract by the bloodstream through phagocytozing immune cells such as macrophages and monocytes. This extragenital route of genital infection has been advocated by the fact that gilts with very limited previous sexual contact were found Chlamydia-positive in genital tract tissues.[32]

In humans, genital chlamydiosis is one of the most significant sexually transmitted diseases worldwide.[46] Infections occur reciprocally during sexual intercourse,[46] but semen has been demonstrated to be the main vector.[47] Moreover, female genital infection has been reproduced in several other species including primates, mice and guinea pigs, either by artificial genital inoculation or mating with infected males.[48-50] In the pig, there is currently no conclusive evidence that female pigs acquire infection through infected semen, either through natural breeding or artificial insemination. However, chlamydial species found in the female genital tract were also found in semen.[30,35] Moreover, if gilts were genitally inoculated with a human genitopathogenic strain of *C. trachomatis*, they developed severe genital pathology.[51] It might thus be suggested, that venereal transmission is possible in principle. It is not known if boars can acquire infection from a genitally infected female during natural mating.

Genital infection and clinical disease

While there was an association between seropositivity and reproductive problems in some studies,[18,38] a recent study failed to confirm this relationship.[29] In contrast to serology, there seems to be a consistency among studies with respect to a positive relationship between the presence of *Chlamydiae* in the reproductive tract and reproductive problems.[29,32,38]

The entire porcine female genital tract seems to be susceptible to chlamydial infection, as co-incubation of genital cells harvested from the cervix, uterine body and uterine horns with *C. suis* strain S-45 led to infection of all three cell lines, albeit with different susceptibility.[52] Generally, chlamydiosis in the female pig has been associated with a wide range of diseases including numerous reproductive disorders such as mummification, [53] abortion,[41,54] perinatal mortality,[55] endometritis,[40,56,57] vaginal discharge,[38] repeat breeding[29,58] as well as poor reproductive performance.[38] In contrast to small ruminants, abortion in swine is a rather sporadic event, of which the pathogenesis is not clear. *Cp. abortus*, *C. suis* and *Cp. pecorum* have been detected in aborted material as single species or in combination. In general, *Cp. abortus* is assumed to be the primary cause of abortion in pigs.[39,54,59] However, *Cp. abortus* does not seem to be obligately pathogenic in swine, as this species was also found in uteri of intact pregnant animals.[60] The fact that different chlamydial species were found in the various oviductal segments (ampulla, isthmus and utero-tubal junction) of repeat breeder pigs suggests that the bacteria may have an effect on oviductal function as seen in women and laboratory animals.[32] For instance, *Chlamydiae* may cause subtle ultrastructural alterations of the oviduct, such as deciliation or cilial dysfunction, which could result in defective ovum capture and transport.[61,62] Chlamydial infection of oviductal cells might also affect secretory patterns of those growth factors and cytokines[63-65] presumably participating in events of fertilization and early embryonic development.[66] Clearly, chlamydiosis in the pig breeding stock is a multifactorial disease with predisposing factors belonging to hygiene and confinement.[38] Moreover, co-infection with other swine pathogens such as PCV-2 may also facilitate the occurrence of chlamydial disease including reproductive failure.[67]

Data on genital disease of boars as the result of a chlamydial infection are patchy, but it seems that the bacteria can cause an inflammation of the accessory glands and the testes.[68,69]

Conclusions

Small ruminants as well as swine can be infected by *Chlamydiae*. While in small ruminants, an infection with *Cp. abortus* is endemic and leads to late-term abortion, infection in swine with *Chlamydiae* (*Cp. abortus*, *Cp. pecorum*,*C. suis*) does not necessarily causes a disease unless exposed to deleterious factor(s). The clinical picture in female swine is variable including abortion, endometritis, vaginitis and vulval discharge. Bucks and rams as well as boars can become infected and subsequenty develop disease, with inflammation seen in the accessory glands, testes, and occasionally the epididymides. Shedding of the bacteria is possible and semen may serve as the vector for *Chlamydiae*.

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Schmallenberg virus in Europe-a review

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Abstract

Schmallenberg virus has spread all over Europe since late summer 2011. It belongs to the family of the Orthobunyaviruses and is transmitted through biting midges and vertical infection. Horizontal transmission is improbable. The virus was mainly detected in ruminants and only in two other species, but typical symptoms such as abortions, stillbirths and malformed newborns only occur in ruminants. Antibodies were also detected in several non-ruminants. There are no indications for pathogenicity in humans. The virus can be diagnosed by real-time PCR, virus isolation or serologically by virus neutralization, indirect immunofluorescence test and ELISA. Two vaccines have been made commercially available in Europe. This review will briefly summarize the current knowledge on the Schmallenberg virus and its disease.

Keywords: Schmallenberg virus, ruminants, Europe

Introduction

In August 2011, there was the first report on an unknown disease that was observed in dairy cows in North Rhine-Westphalia (Germany). A similar case was reported from the Netherlands at the same time. Clinical signs that were associated with the disease were non-specific such as fever, diarrhea and decreased milk production.[1] Later on, lambs were born that showed different degrees of malformation.[2] Laboratory tests were all negative for classical bovine endemic and emerging viruses such as pestivirus (BDVD), bovine herpesvirus 1 (BHV1), foot and mouth disease virus (FMDV), bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), Rift Valley fever virus (RVFV), and bovine ephemeral fever virus (BEFV). At the same time, comprehensive investigations performed at the Federal Research Institute for Animal Health in Germany that also included a metagenomic analysis revealed that the disease was caused by a new virus belonging to the family of Orthobunyaviruses.[3] Because the virus was found in a pooled blood sample obtained from three sick cows from a dairy farm near the German town Schmallenberg, North Rhine-Westphalia, the new virus was named "Schmallenberg virus".[3] Since its first detection, the virus quickly spread all over Europe. In Germany, for instance, Schmallenberg virus-associated disease which is considered a notifiable animal disease in ruminants, was observed on 1,478 cattle farms as well as 973 sheep and 53 goat flocks until March 2014.[4] This contribution will briefly review the disorder.

Epidemiology

Schmallenberg virus is part of the Simbu serogroup of the genus *Orthobunyavirus* within the family *Bunyaviridae*. Akabane virus,[5,6] Aino virus[7,8] and Shamonda virus[9,10] cause similar symptoms as Schmallenberg virus (see below) and are widespread in Oceania, Australia, Africa, and Asia.

Clinical signs have not been reported in non-pregnant adults with the exception of one report of sudden astasia and leukopenia in a naturally infected dairy cow. In naturally infected pregnant cattle and sheep, Aino virus has been associated with stillbirths, premature births, and birth defects including arthrogryposis, scoliosis, sunken eyes, cataracts, maxillary retraction, and dental irregularities. Some calves may have a domed head from hydranencephaly and cerebellar hypoplasia.

Schmallenberg virus is relatively liable in the environment or the vectors, as it does not survive outside its natural hosts for a long time. Also, it is susceptible to standard disinfectants such as 70 % ethanol.

It seems that Schmallenberg virus is almost completely adapted to domestic ruminants, as the virus itself was almost exclusively found in cattle, sheep and goats by either PCR or virus isolation. There are only two reports on virus positivity in other than the aforementioned species, i.e. in a dead elk calf[11] and in one dead puppy.[12]

There are a number of other ruminants and also non-ruminant species that were found serologically positive including roe deer, fallow deer, red deer, sika deer, chamois, bison, moufflon as well as alpaca, dog and wild boar.[11-20]

Schmallenberg virus spread all over Europe within a very limited time period i.e. 2 1/2 years.[4] The list of countries that are positive includes Germany,[3] the Netherlands,[2] France,[21] Belgium,[22] Luxembourg, Austria, Switzerland,[23] Italy,[24] Poland,[11] Czech Republic, Spain,[25] Denmark,[26] the UK,[27] Ireland,[28] Norway,[29] Finland, Sweden, Estonia, Latvia, Hungary, Slovenia, Romania, Croatia, Serbia, Turkey,[30] and Greece.[31] Other neighboring countries are most likely also positive, but have yet not been sampled.

The prevalence of Schmallenberg virus infection in dairy herds is high. Studies conducted in the Netherlands and Belgium in 2012 revealed a prevalence at farm level of 95.5 % and 99.76 %, respectively, in 2012.[32,33] Moreover, the intra-herd-prevalence was 86.3 % in the Dutch study.[32] A high prevalence of 97.1% was also found in sheep flocks in the Netherlands.[34] Within a flock, 58.7-84.31% of the sheep were found seropositive.[34,35] Goats were found less often infected; the intra-herd-prevalence was 40.68 % in Belgium, 36.7 % in the northwest part of Germany and 43.8 % in Lower Saxony, Germany.[34-36]

Schmallenberg virus is transmitted by biting midges (*Culicoides* spp.). The virus was found in several *Culicoides* spp. (i.e. *C. obsoletus complex, C. dewulfi, C. chiopterus, C. scoticus, C. punctatus*) in Denmark, the Netherlands, Belgium and Poland.[26,37-40] A horizontal transmission through animal contact is unlikely. For instance, in one experiment where two cows were orally inoculated with virus did not result in an infection. Also, sentinel cattle kept in the same compartment remained virus- and sero-negative.[41] Schmallenberg virus can also be detected in semen of infected bulls.[42-44] In a recent study performed by Wim van der Poel et al,[44] two bulls were inoculated subcutaneously with a Schmallenberg virus isolate. Virus RNA was then isolated from blood samples two to four days after inoculation. In semen, the highest amounts of virus were observed on days four to seven after inoculation, followed by intermittent shedding during the next two to three weeks. However, the clinical relevance of virus transmission by semen and artificial insemination still needs to be determined.[43]

Clinical signs and pathology

Generally, there are only weak clinical signs in the adult animal that are associated with the infection. In adult cows signs are rather inconspicuous or nonspecific such as fever (up to 41°C), decreased milk production and diarrhea.[1] The signs last for up to six days (concurrent with the time period of viremia that also lasts for up to six days).[45] Infections occur seasonally with higher incidences in summer and autumn when midges are most active. Up until now, no clinical signs have been described in goats and sheep. An infection experiment performed with 30 adult sheep resulted in a mild or subclinical disease course in only a few animals (diarrhea in one sheep; snotty nose in two sheep).[46] Overall, the situation resembles that of Akabane virus infection which also causes only mild clinical signs or is subclinical in ruminants.[5]

Vertical transmission of Schmallenberg virus and infection of the fetuses can lead to embryonic mortality, malformed newborns, abortions and stillbirths. Virus has been isolated from mummies.[2] However, there is a critical or susceptible window in gestations where an infection has to happen for malformations to occur. In sheep this window is between days 28 and 60 of gestation;[47] for cattle it is between day 80 and 150.[6] For Schmallenberg virus it is yet not known what happens to the embryos or fetuses if an infection occurs prior to, or after the aforementioned windows. However, assuming that the pathogenesis of Schmallenberg virus infection is similar to Akabane virus infection, infections outside the aforementioned windows may still lead to embryonic/fetal death, but malformations may then be rare or absent.[48]

The predominant malformation in calves, lambs and kids is the "arthrogryposis-hydranencephalia-syndrome". Generally affected newborns do show one or more of the following malformations: arthrogryposis, vertebral malformations, brachygnathia inferior, ankylosis, torticollis, and scoliosis. In addition, malformations of the central nervous system (CNS) can occur, including

hydranencephaly, porencephaly, hydrocephalus, cerebellar hypoplasia, and micromyelia.[49-51] Most of the malformed newborns are dead at birth. Those that are born alive are usually nonviable and have to be euthanized immediately. Furthermore, especially malformations of the limbs can cause dystocia that then require obstetrical intervention. Also, due to limb malformations perforations of the uterine wall during labor are possible.[52] If the CNS is additionally affected, newborns may also show a decreased or missing sucking reflex and develop locomotion problems.

Diagnosis

The Schmallenberg virus can be detected by real-time RT-PCR or by virus isolation using different cell lines.[3] The best time to isolate the virus is during the period of viremia (i.e. within the first week of infection) using EDTA-blood or serum. Also, different tissue specimens such as brain, spleen, meconium and amniotic fluid from stillborn or malformed newborns are feasible.[53] Virus specific antibodies can be detected by virus neutralization, indirect immunofluorescence test or by ELISA (there a several commercial antibody ELISA-Kits available).

Zoonotic potential

Susceptibility of Schmallenberg virus for humans is still patchy. Available data suggest, however, that the risk of a transmission from animals to man is unlikely. For instance, there are two recent studies conducted in Berlin, Germany, by the Robert Koch Institute, and in Bilthoven, The Netherlands, by the National Institute for Public Health and the Environment.[54,55] In these studies, personnel working in infected sheep and goat flocks and cattle barns, as well as the responsible veterinarians, were tested for antibodies against the virus. None of the tested people were positive for virus specific antibodies.

Control and prophylaxis

There is currently no specific treatment against the Schmallenberg virus. There are two vaccines (Bovilis® SBV [Intervet UK Ltd, Milton Keynes, Buckinghamshire, United Kingdom], SBVvax [Merial SAS, Lyon, France]) that are available in two European countries, i.e. in the United Kingdom and in France, to be used in sheep and cattle. Both vaccines contain inactivated virus. According to the labels, sheep at four months or older are to be vaccinated only once while in cattle, two vaccinations are required at an age ≥ 2 months and again three weeks later. A clear antibody response can be usually observed three weeks after first (sheep) or second (cattle) vaccination. While it is not yet known how long protection may last, the general assumption is for at least 12 months. To the author´s knowledge there is no commercial vaccine available for use in goats.

Another preventative measure is to use repellents and pyrethroides directed against the vector, i.e. biting midges, thus reducing the risk of an infection with the virus. Another but probably only theoretical approach is to breed the animals in colder times of the year (i.e. in the late autumn or winter time) to avoid having the dams pregnant during the time of gestation when the fetuses are susceptible to an infection.

Summary and conclusion

Up until now clinical disease caused by Schmallenberg virus has only been seen in ruminants. Main economic losses result from the infection of pregnant animals leading to stillbirths, abortions, malformations and embryonic death. Since the virus is transmitted by biting midges, preventive measures that would completely protect from an infection are almost impossible to be implemented. Currently, vaccination is the best and only effective way to reduce clinical disease and thus reduce economic losses due to an infection. In addition, repellents and pyrethroides may help as a vector control measure.

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Bovine abortion: diagnostic methods

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Bovine abortion continues to result in severe economic losses for beef and dairy producers through out the world. The average herd abortion rate reported in various studies in the literature varies from 2% to 12%, with much of the variation due to differences in case definition (what constitutes an abortion), methods of data recording, management styles, geography and prevalence of bovine abortifacients. It is interesting to note that in spite of an abundance of vaccine products for abortion prevention, these figures remain virtually unchanged. The endless battle for prevention of abortion losses continues. This presentation will discuss the methods of diagnosis for the common causes of bovine abortion. Possible ruleouts for bovine abortion will also be covered.

Success rates for abortion diagnosis are often very low averaging around 30% in most laboratories. Many cases of non-infectious abortion will go undiagnosed. The Animal Disease Research and Diagnostic Laboratory (ADRDL) at South Dakota State University has adopted a uniform approach for dealing with cases of bovine reproductive failure that is unique in the field of veterinary diagnostic medicine. Unique does not necessarily mean the only way or the best way. All cases of reproductive failure submitted to ADRDL are assigned to one pathologist (i.e. a reproductive disease specialist) who acts as case coordinator until the case is completed. The most obvious advantages of this system are consistency and accountability. As the reproductive disease specialist, I encourage practitioners to feel free to contact me with questions and special needs on difficult cases. I will not always have an answer, but I will always try to lend as much assistance as is possible.

Submission of case material for abortion diagnosis is a straightforward process. However, a high percentage of submissions every year are incomplete, reducing the already limited diagnostic success rates. Start every abortion investigation by obtaining a complete history including a description of on farm management practices, vaccination protocols and an evaluation of recent on farm events that may have predisposed the cow to abortion disease. Previous disease problems, new purchases and nutritional programs should be recorded. A complete history is received with probably less than 10% of submitted cases.

An intact fetus and placenta are desirable for diagnostic evaluation. If the fetus is large and shipping costs become prohibitive, a complete necropsy should be performed on the farm or at the veterinary clinic. A necropsy is not a difficult task; however, diagnostic success will often hinge on the submission of appropriate samples. Encourage producers to attempt to recover placenta. Often the placenta is retained and must be retrieved from the cow, or is left lying on the ground or lost to scavengers. The bottom line is that the placenta is the most important tissue for abortion diagnosis and without it, the odds of an etiologic diagnosis are reduced. Educate the client as to the importance of the placenta for diagnostic success in abortion cases.

The procedure for a necropsy on bovine fetuses relatively easy and requires no specialized equipment. A sharp knife (Chicago cutlery 62S), pruning shears, meat saw or back saw, scissors, forceps, sterile sample bags (Whirl-Pacs®, Nasco, Ft. Atkinson, WI) and a leakproof container of 10% buffered neutral formalin is all that is required. The pruning shears are excellent for removing the rib cage. The saw is useful for removal of the brain. The secret to success in performing necropsies is repetition and observation. Collect appropriate samples the same way every time and you will not leave out something that may be useful for diagnosis. Record you observations. Package samples so that cross-contamination between tissues will be minimized. If you package lung and placenta in the same bag, and the placenta was laying in the mud for hours, we probably will culture organisms from the mud instead of the more slowly growing abortion pathogens. Fetal stomach content and thoracic fluid are collected with a sterile 18-gauge needle and syringe. Ideally, you should transfer the fluid to a sterile tube and avoid the risks associated with sending syringes with attached needles. Whirl-Pac® bags that are properly sealed will not routinely leak during transit. Postal authorities are becoming increasingly alarmed at the possible risks associated with leaking packages in the mail system. Use common sense and good packing materials to

avoid these risks. An insulated container system is available for purchase from many laboratories and will aid in the shipment of fresh samples to the diagnostic laboratory. Take into consideration the ambient temperature when submitting samples. Avoid weekend stays in the post office if possible. Temperatures over 100°F tend to adversely affect an already autolysed fetus. Conversely, histologic examination of frozen fetuses is also less than ideal.

When a case submission is received at the diagnostic laboratory, samples are processed and submitted to the appropriate laboratories for further diagnostic investigation. For bacteriology, fetal lung and stomach content are our tissues of choice. Occasionally, liver and placenta will be cultured. Routine samples are cultured on Columbia agar with 5% sheep blood. The plates are incubated at 37°C in an atmosphere containing 10% CO_2 and routinely examined at 24 hour intervals. When numerous similar colony types are observed, the organism is isolated and characterized by biochemical reactions. Stomach content is often examined by darkfield microscopy for preliminary identification of *Tritrichomonis fetus*, *Campylobacter sp.* and leptospirosis. For *Campylobacter sp.*, special media with added antibiotics are used for primary isolation. Specific atmospheric conditions are also required. *Campylobacter jejuni* is the most common species isolated from bovine abortions.

Mycotic abortions are best diagnosed by examination of affected placentas. Gross examination of the placenta will often lead to a presumptive diagnosis based on the characteristic thickening in intracotyledonary spaces. The placenta should be spread out flat and extra debris should be removed for a complete examination. A fluorescent dye called calcofluor white M2R is routinely used to detect fungal elements in placental scrapings and impression smears. Routine fungal cultures are plated on appropriate media including Sabouraud's and mycobiotic agar, and incubated at 25°C for seven days.

Fluorescent antibody (FA) techniques and polymerase chain reaction (PCR) test are routinely used to detect infectious bovine rhinotracheitis virus (IBR), bovine viral diarrhea virus (BVD) and leptospirosis. Virus isolation procedures are routinely performed on all submitted cases that are considered suitable for examination. Fetal tissues including lung, spleen, kidney, heart and liver are pooled and inoculated on susceptible fetal bovine cell lines. Samples of placenta are usually cultured separately. Cultures are evaluated for cytopathic effect (CPE) after seven days. Cultures free of CPE are passed to fresh cells and observed for an additional seven days. Isolates are identified by FA techniques. Infectious bovine rhinotracheitis virus tends to grow rapidly in cell culture and is usually easy to diagnoses on FA. In contrast, BVD is much more difficult to culture and may not be isolated until after the first passage.

Tissues for histologic examination are submitted in 10% buffered neutral formalin. Adequate formalin and thin slices of tissue (<0.5 cm) will insure adequate fixation of fetal tissue. Formalin-fixed tissues are sectioned at 5 μm and stained with hematoxylin and eosin (H&E) and examined microscopically. Tissues including placenta that may be involved in a mycotic infection are stained with Gomori's methenamine-silver nitrate. Keep in mind that autolysis of fetal tissue is often advanced if the fetus was retained in-utero for a prolonged period.

Immunocytochemistry procedures on formalin fixed tissues are routinely available for BVD and *Neospora caninum*-like protozoal agent. This technique may prove to be of considerable value for cases in which fresh tissue is unavailable for examination. Additional tests are currently being added for common abortion causing agents.

Serology testing is often of little value in individual animal cases; however, serologic profiling for common abortion agents may be of value in a herd situation. Serologic tests are available for IBR, BVD, leptospirosis, brucellosis, and neosporosis.

Common causes of bovine abortion

Infectious

Epizootic (>20%)	Sporadic: High Incidence	Sporadic: Low Incidence
Neospora caninum-like protozoa	*Neospora caninum*	*Campylobacter*
Infectious bovine rhinotracheitis*	*Salmonella* sp.	Listeriosis
Leptospirosis*	IBR	Chlamydia
Brucellosis**	BVD	BVD
Epizootic bovine abortion	*T. pyogenes*	*T. pyogenes*
	Mycotic	Mycotic
		Trichomoniasis
		Bacillus sp.
		E. coli
		Pasteurella sp.
		DN 599

*Uncommon in vaccinated herds
**Eradication program

Epizootic abortion is relatively uncommon - most epizootics investigated by our laboratory are associated with *Neospora* in dairy herds; historically epizootics have been most common with IBR. Sporadic abortions are most often associated with *Trueperella pyogenes* and mycotic abortion with *Aspergillus fumigatus*.

Non-infectious

Very little information is available concerning the causes and incidence of non-infectious abortion.

A. Genetic
   a. lethal genes
   b. chromosomal abnormalities
B. Nutritional-commonly associated with infertility and neonatal mortality
   a. chronic starvation
   b. vitamin A deficiency
   c. iodine deficiency
   d. manganese deficiency
   e. vitamin E/ selenium deficiency
C. Toxic plants
   a. broomweed (*Gutierrezia* spp.)
   b. locoweed (*Astragalus* and *Oxytropis* spp.)
   c. pine needles (*Pinus ponerosa*) incidence may vary within a herd depending on exposure and stage of gestation
   d. ergot (*Claviceps purpurea*)
   e. narrowleaf sumpweed (*Iva angustifolia*)
D. Toxins
   a. nitrates/nitrites
   b. mycotoxins
   c. endotoxins

E. Hormonal
   a. progesterone deficiency
   b. estrogens
   c. corticosteroids
F. Physical
   a. trauma
   b. umbilical cord torsion
   c. stress, surgery (xylazine), transportation, systemic disease
G. Miscellaneous
   a. twinning (common cause in dairy herds)
   b. hyperthermia
   c. allergies and anaphylactic reactions

Bovine abortion-sample submission

Fetus and placenta:
The entire fetus and placenta, chilled, not frozen, are the preferred specimens when transportation can be arranged. When the entire fetus cannot be submitted to the laboratory, the following specimens are the minimum if a complete examination is to be done:

Formalin fixed	Fresh (chilled)
lung	lung *
liver	liver
kidney	kidney
spleen	spleen
heart	heart
brain (1/2)	brain*
skeletal muscle (tongue, diaphragm)	placenta*
placenta (grossly examine for focal lesions)	

Also collect:
stomach content-1-3 ml in sterile disposable syringe**
thoracic fluid or heart blood from fetus-3-5 ml in sterile disposable syringe**

Maternal blood should be collected and 3-5 ml of serum should be separated from the clot. Serology on individual animals is often unrewarding. Samples should be saved for further evaluation in a whole herd profile at a later date, if not submitted with the initial case.

Put the fresh tissues in sterile bags, and chill or freeze if delivery to the laboratory will be prolonged. Put formalin-fixed tissue in an unbreakable, leak-proof container. Label samples accordingly. Ship in an insulated container with enough ice packs to maintain refrigerated conditions until arrival at the laboratory.

*package these tissues in separate Whirl-Pacs®
**transfer to sterile tube if possible

Do not hesitate to contact the laboratory for assistance in sample collection or submissions procedures! Procedures will vary from laboratory to laboratory.

Ovine infectious abortion

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Infectious abortion in sheep flocks is a serious, widespread disease problem in North America. It is important for sheep producers and their veterinarians to understand the circumstances under which abortion diseases occur, so that effective preventive health and management programs can be implemented to minimize economic loss from these diseases. The following outline should provide you with sufficient information about the abortion diseases to help you formulate a disease control/prevention plan that should work for the majority of operations that you work for. Also keep in mind that a 0% abortion rate is probably not an obtainable goal.

Campylobacteriosis (Vibriosis)

Introduction

Campylobacteriosis (Vibriosis) is an acute contagious disease of pregnant ewes. The disease causes extensive financial loss to the sheep industry.

Etiology and pathogenesis

Campylobacteriosis is caused by the bacteria *Campylobacter fetus* ss. *fetus* and *Campylobacter jejuni*. Transmission involves ingestion of the bacteria from feces or fluids from aborting ewes, and uterine localization with subsequent abortion is an accidental outcome of a brief period of bacteremia (bacteria in the blood stream) in non-immune sheep. *Campylobacter jejuni* is a common inhabitant of the intestinal tract of normal cattle, dogs, cats, and rodents although significance of each of these reservoirs is unknown. Campylobacter may be spread from infected farms to non-infected farms by purchase of carrier sheep, birds and other animals, particularly coyotes and dogs. Stress from other abortive agents, weather, nutrition and overcrowding can trigger *Campylobacter* abortions.

Clinical signs

Campylobacter causes abortion during the last trimester of pregnancy or birth of dead or weak lambs. Most aborting ewes recover but some die from retention of dead lambs, infection of the uterus, and peritonitis. Abortion rates vary from 5% to 70% in a natural outbreak.

Diagnosis

Abortions resulting from *Campylobacter* are usually visually indistinguishable from other causes of abortion. Consequently, laboratory diagnosis is necessary. Aborted fetuses and placentas should be submitted to a diagnostic laboratory. Concurrent infections with other agents such as Toxoplasmosis are possible.

Treatment and prevention

Rapid diagnosis is critical. The predominant *Campylobacter* sp. isolated in the upper Midwest is *C. jejuni*. Essentially all of these isolates appear to be resistant to tetracycline in vitro and in vivo. Once an outbreak of *Campylobacter* occurs in the flock, I would still recommend starting antibiotic therapy until the sensitivity results are known. In years previous, and to some extent today, some people will recommend vaccination in the face of an outbreak. Immunity to *Campylobacter* is strain specific and there are probably numerous strains circulating in a large population of sheep. Within an open flock, adding new sheep may mean adding a new strain. Prevention of *Campylobacter* sp. by vaccination of ewes at breeding may help, but remember, protection from one stain to another is limited. Autogenous vaccines are becoming more common in large flocks. Avoid feeding ewes on the ground. During an outbreak, spread of the disease can be minimized by burning or burying all aborted fetuses and placental membranes. In addition, aborting ewes should be isolated until all uterine discharge has ceased.

Zoonotic risks

Campylobacter jejuni is one of the leading causes of human food poisoning. Clinical signs include a severe diarrhea. *Campylobacter jejuni* is transmissible from infected ewes, aborted fetuses, and placental membranes to humans.

Toxoplasmosis

Introduction

Toxoplasmosis is an infectious disease that occurs sporadically, usually in farm flocks, causes considerable economic loss to the sheep industry.

Etiology and pathogenesis

Toxoplasmosis is caused by a coccidial organism (*Toxoplasma gondii*) that is carried by domestic cats. Young cats excrete the organism in the feces. Transmission of the infection from cats to sheep occurs when the sheep feed becomes contaminated with feces from infected cats. Ingestion of the contaminated feed causes infection in sheep.

Clinical signs

Ewes infected with *T. gondii* are asymptomatic. In a rare case, a ewe may show signs of encephalitis, including walking in circles, uncoordinated movements, muscular rigidity, prostration and impaired vision. Ewes with uterine toxoplasmosis may abort during the last month of pregnancy or give birth to dead or weak lambs. The weak lambs are listless, uncoordinated and usually die from starvation. Abortion usually occurs about four weeks after infection in experimental cases. Mummified fetuses are often a feature of toxoplasma infection during gestation.

Diagnosis

Toxoplasmosis is diagnosed by laboratory examination of fetal blood and thoracic fluid by indirect fluorescent antibody procedures for detection of antibody to *T. gondii*. Fetal tissues and placenta from aborting ewes are examined for characteristic gross and histologic lesions. Occasionally, serologic screening of the ewe flock may be necessary to make this diagnosis in an endemic flock.

Treatment and prevention

There are limited data that feeding monensin (Rumensin®) and decquinate (Deccox®) will reduce the severity of placental infection associated with toxoplasmosis in pregnant ewes. The medication must realistically be fed throughout gestation. Experimentally, lasalocid (Bovatec®) has been shown to have no effect on *T. gondii*. Limiting cat populations and avoiding contamination of sheep feed and water with cat feces provides satisfactory control of the disease. Shedding of this organism is most common in young cats or cats with litters. Mature cats that have been spayed or neutered present limited risks for transmission of infective oocysts. Currently there is a modified live vaccine in use in England, although no products are approved for use in the United States.

Zoonotic risks

Toxoplasmosis is a significant zoonotic risk for humans. Pregnant women are at particular risk since toxoplasmosis causes encephalitis in the fetus. Humans usually contract this disease from exposure to feces from infected cats shedding oocysts.

Enzootic abortion of ewes; Chlamydiosis

Introduction

Enzootic abortion of ewes (EAE) is a highly contagious zoonotic disease that causes severe economic loss to the sheep industry in many geographic areas.

Etiology and pathogenesis

Enzootic abortion of ewes is caused by an intracellular (lives most of its life cycle inside the cell) microscopic organism, called *Chlamydophila abortus* (old name *Chlamydia psittaci*). The transmission of EAE from infected sheep to susceptible sheep occurs at the time of parturition and abortion. Some data suggest that possible transmission may occur through a fecal oral route or oral contact with uterine fluids in cycling ewe lambs. The aborted fetus, placenta, and uterine fluids contain large numbers of infective organisms allowing for rapid transmission of the disease. Transmission also probably occurs to some degree through feed or water contaminated by aborting ewes and subsequently ingested by susceptible ewes. Crowding in lambing facilities may also causes spread of EAE through the respiratory tract.

Clinical signs

Infection is most often asymptomatic. Infection of ewes between 30 to 120 days of lambing usually results in abortion or birth of weak lambs, while infection during the last month of pregnancy may result in latent infection and possibly abortion during the next pregnancy. Ewes may die as a result of secondary bacterial infection of the uterus (retained placentas etc.).

Diagnosis

It is important to obtain laboratory confirmation if EAE is suspected. The aborted fetus and the placenta are required for laboratory examination. A laboratory diagnosis is sometimes difficult and may require submission of several aborted fetuses with placentas if the sample quality is poor. Diagnosis without submission of placenta is usually unrewarding.

Treatment and prevention

Outbreaks of EAE are treated with tetracycline antibiotics in the feed at the rate of 500 mg to 700 mg per head daily. Treatment with long-acting tetracycline may extend the duration of a threatened pregnancy. The net result of tetracycline treatment is suppression chlamydial multiplication. Antibiotic therapy will not reverse the damage to the placenta that is already done. Antibiotic therapy as a routine procedure should be evaluated in light of the situation in each individual flock. Prevention of EAE has been attempted for many years through vaccination. Currently available products are probably minimally effective. Routine administration of feed grade tetracycline during the last trimester of gestation (five days on, two weeks off) has significantly reduced the incidence of *Chlamydophila abortus* in the upper Midwest. Pregnant ewes should not be fed on the ground, thus preventing feed contamination by aborting ewes. Aborting ewes and weak lambs should be kept isolated from other ewes and lambs until all uterine discharges cease and surviving lambs are normal. Aborted fetuses, weak lambs that have died and placentas should be burned or buried. Infected premises should be cleaned and disinfected as circumstances allow.

Zoonotic risks

Chlamydophila abortus is a potentially serious threat to pregnant women and any individual that is immunosuppressed (cancer therapy, steroids, HIV, etc.). Hepatitis and severe flu-like symptoms have been reported. Abortion in pregnant women is a real threat.

Salmonellosis

Introduction

Salmonellosis is an acute contagious disease. Outbreaks of the disease occur sporadically and can result in severe economic loss.

Etiology and pathogenesis

Salmonellosis is caused by several species of *Salmonella* bacteria. *Salmonella* are harbored by adult carrier ewes that shed the bacteria into the environment through the feces. Ewes develop resistance with age or a result of exposure to the bacteria. First lambing ewes, having little or no resistance to the bacteria, are susceptible to the infection. Disease occurs as a result of non-immune ewes ingesting *Salmonella* through contaminated feed or water. Infected cattle (particularly calves) can be a source of *Salmonella* infection for sheep.

Clinical signs

Affected ewes usually develop high fever and severe diarrhea, are depressed and off-feed, abort and may die. Others may show minimal clinical signs. Ewes that survive have much less milk for their lambs. Lambs that are born alive from infected ewes are usually weak and may die.

Diagnosis

Diagnosis of salmonellosis is accomplished by bacterial culture from tissue collected from aborted fetuses and tissues from the affected ewe.

Treatment and prevention

Several antibiotics have been used to treat salmonellosis with generally poor results. Aggressive antibiotic therapy may be required to limit death loss in the ewe flock. Prevention of the disease is related primarily to management since vaccines have limited use against salmonellosis.

Zoonotic risks

Salmonella bacteria are very infective for humans. Extreme care should be exercised when handling infected tissues.

Listeriosis (circling disease)

Introduction

Listeriosis is an acute, infectious, but non-contagious disease that occurs sporadically in sheep flocks. The disease causes considerable economic loss in affected flocks.

Etiology and pathogenesis

Listeriosis is caused by the bacteria *Listeria monocytogenes*. The organism lives in the environment and most commonly causes disease through the feeding of improperly ensiled or spoiled silage or spoiled hay. Listeriosis usually occurs as encephalitis but may cause abortion.

Clinical signs

Ewes with the neurological form of listeriosis become depressed and disoriented. Affected sheep may walk in circles. A head tilt and facial paralysis on one side is commonly observed. The mortality rate of sheep affected is usually high. The uterine form of listeriosis causes abortion in late pregnancy. Most aborting ewes recover from the infection.

Diagnosis

Diagnosis of listeriosis is made on the basis history and clinical signs. Confirmation of the diagnosis is accomplished by laboratory isolation of the causative bacteria from the affected animals. Numerous bacteria are present in all tissues on histologic examination.

Treatment and prevention

There is no proven effective treatment for listeriosis. Prevention of the disease is accomplished by feeding good quality silage. When an outbreak of listeriosis occurs, discontinue feeding silage, isolate affected sheep. Properly dispose of sheep or fetuses that have died from listeriosis.

Zoonotic risks

Human cases of listeriosis have occurred from handling infectious tissues and from inhaling dust from dried bedding in contaminated lambing sheds.

Miscellaneous etiologies

Border disease virus, Cache Valley fever, *Flexaspira rappani*, *Yersinia enterocoliticus*, *Bacillus* sp. *E. coli* and several viruses are occasionally associated with abortion in sheep flocks. The significance is usually minimal.

Key management practices to prevent/control abortion diseases

1. Minimize stress and overcrowding during late gestation.
2. Maintain a closed flock.
3. Do not feed pregnant ewes on the ground (EAE, campylobacterosis, and salmonellosis).
4. Limit cat population and protect feed from cat fecal contamination (toxoplasmosis).
5. Keep first lambing ewes separate from mature ewes when confined at lambing (salmonellosis).
6. Isolate sick, infected (aborting) ewes to avoid transmission of disease (EAE, campylobacterosis, and salmonellosis).
7. Do not allow pregnant ewes to drink ground water in confinement which is subject to fecal contamination.
8. Dispose of aborted fetuses, placentas, and dead lambs by incineration or deep burial if possible.
9. Utilize the assistance of a competent sheep veterinarian and diagnostic laboratory to establish accurate diagnosis and effective plan of action when abortions begin.
10. Vaccination and antibiotic therapy are valuable management tools. They will not necessarily eliminate the problem. Each product should be evaluated for efficacy. Some products currently on the market are of limited value.

Sample submission for diagnostic evaluation of ovine abortion

1. Fresh chilled fetuses with placenta are the specimen of choice. Frozen lambs are of limited diagnostic value.
2. If a necropsy is performed at the farm or clinic, submission of the following tissues is recommended:
   a.) fresh and formalin fixed (10% buffered neutral formalin) tissues including lung, liver, kidney, spleen, heart, brain and PLACENTA.
   b.) fetal thoracic fluid or heart blood (for indirect FA's for toxoplasmosis and *Chlamydia*)
   c.) fetal stomach content (bacterial culture)
3. Serum from the ewe is usually of little value early in an abortion workup. It may be a good idea to bank serum from affected ewes if the need arises at a later date.
4. An adequate history should include percent or number of ewes affected, breed, age of affected ewes, parity, nutritional status, previous disease conditions, vaccination history, antibiotic therapies and environmental conditions as they may apply to the existing problem.

Rediscovering the silent enemy in cattle reproduction health: from the latest findings about sexually transmitted trichomoniasis and campylobacteriosis to the current control and future therapeutics

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The objective of this review is to discuss sexually transmitted diseases (STDs) caused by *Tritrichomonas foetus* (*T. foetus*) and *Campylobacter fetus* (*C. fetus*) subsp. *venerealis* focusing on prevalence, pathogenesis in cows and bulls and diagnosis. Later, we examine the research on prophylactic systemic immunization of bulls and cows with antigens of *T. foetus* and *C. fetus* subsp. *venerealis* and the efficacy in protecting, preventing or sometimes even clearing pre-existing infections in the genital tract. An analysis of the latest advances in bovine trichomoniasis and campylobacteriosis may establish common knowledge to pursue united efforts for further control of STDs.

Keywords: Cattle, sexually transmitted diseases, trichomoniasis, campylobacteriosis, vaccines

What are bovine sexually transmitted diseases?

Bovine STDs comprise trichomoniasis caused by flagellated obligated extracellular protozoon *T. foetus* and campylobacteriosis (i.e., bovine vibriosis) caused by gram-negative bacterium *C. fetus*. Two species of *C. fetus* are relevant in cattle health: *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis.*[1,2] *C. fetus* subsp. *venerealis*, including the biotype *intermedius* inhabits exclusively the genital tract of cattle and is the causative agent of campylobacteriosis.[1-3] However, *C. fetus* subsp. *fetus* may also provoke sporadic genital infection and abortion as it inhabits the intestine but it can colonize the genital tract via ascending genital infections or venereal route.[4,5] Coitus is the primary route of transmission of *T. foetus* and *C. fetus* subsp. *venerealis,* although *T. foetus* and *C. fetus* subsp. *venerealis* can survive in raw and processed bull semen and be also spread via artificial insemination (AI).[5,6]

Both STDs cause early pregnancy loss, infertility and late abortion. The STDs are responsible for important economic losses due to reproductive losses, including poor conception rates (from slightly subnormal to 50 percent or lower), reduced calf crops, increased days-to-conception, extended calving seasons, cost of replacement bulls, loss of genetic potential due to culling, and subsequently lighter weaning weights. A study of the impact of bovine trichomoniasis predicted a reduction of 14 to 50% in annual calf crop, a prolonged breeding season, a reduction of 5 to 12% in weight gain in the suckling / growing period, a reduction of 4 to 10% in pounds of marketable calf crop at weaning, a reduction of 4 to 10% in monetary return per calf born, and a substantial reduction of 5 to 35% in the return per cow confined with a fertile bull.[7]

Are bovine STDs still important and prevalent?

Bovine STDs are distributed worldwide with high disease incidence in developing countries, where natural breeding of cattle is widely practiced. However, STDs are also still endemic in developed countries, where natural breeding is commonly practiced in beef herds.[8] Latest studies reported bulls infected with *T. foetus* in herds from countries around the world, including USA,[9-11] Argentina,[12] Spain,[13-15] Australia,[16] and Transkei.[17] Likewise, bulls infected with *C. fetus* subsp. *venerealis* have been reported during the last decades in USA,[18] Australia,[16] Great Britain,[19] Colombia,[20] Tanzania,[21] Nigeria,[22] Canada,[8,23] Argentina[24,25] and Transkei.[17] Recent sporadic outbreaks of bovine abortions associated with *C. fetus* have been reported in developing areas of Africa, Asia, and South America and even in developed countries of Europe, Oceania and North America.[8] Most importantly, infections by *T. foetus* and *C. fetus* subsp. *venerealis* are clearly underestimated in many regions, including in North America, because there is no global governmental monitoring programs for STDs, lack of reports in most countries and/or testing is limited for some animal categories or situations. For instance, trichomoniasis testing in several US states is required only for bulls 18 months of age and older sold through a public sale yard (there are no federal requirements in place), ignoring private treaty sales and the presence of the disease in

cows or herds that do not trade livestock. Lastly, STDs are intrinsically associated with cattle production systems deficient in management and veterinary assistance which makes it more difficult to identify and control and; to make matters worse, they usually lack the proper laboratory facilities for the routine diagnosis. Thus, areas with extensive cattle raising and natural breeding, commonly found in Latin America, Africa, Asia, western North America and some countries of Europe, still have a high prevalence of trichomoniasis and campylobacteriosis of unknown magnitude, spread and economic importance.

What is known about bovine STDs?

Bulls-the silent and permanent carrier

Tritrichomonas foetus. *T. foetus* persistently and asymptomatically colonize the epithelium of the prepuce, penis, and rarely the urethral orifice of bulls. One study showed that *T. foetus* was cultured from the preputial and penile epithelial surfaces of 24/24 bulls infected with *T. foetus* (15 naturally and 9 experimentally), as shown by culture of smegma samples, but from the urethral orifice of only 4/24 infected bulls.[26] Likewise, in other study with 24 bulls naturally infected with *T. foetus* and carriers at slaughter or within four months before, as shown by smegma culture, *T. foetus* was detected by immunohistochemistry on the epithelial surface of preputial (5/24 bulls) and penile (14/24 bulls) crypts, but never in the penile and prostatic urethra, seminal vesicles, prostate or epididymis.[27] However, it appears that *T. foetus* is restricted to mucosal surfaces and incapable of invading tissue. No whole trichomonads were found in genital tissue sections by immunofluorescence with anti *T. foetus* immunoglobulins or by histological staining in 24 *T. foetus*-infected bulls.[26] Similarly, trichomonad antigens but not whole trichomonads have been detected by immunohistochemistry a few cell layers deep and occasionally below the basement membrane in the preputial and penile epithelial crypts of 24 *T. foetus*-infected bulls.[27] The superficial location of *T. foetus* suggests that antigen presenting cells (APCs) could capture *T. foetus* antigens from genital surfaces by using their dendritic projections or as a result of contacting with genital epithelial and subepithelial cells, which have shown to take up *T. foetus* antigens in bulls[27] and interact with stromal APCs, at least in female rats.[28]

Experimental and natural genital infections with *T. foetus* in bulls caused no clinical signs or gross pathological changes.[26] The microscopic response in the prepuce and penis of bulls infected with *T. foetus*, characterized by infiltration of intraepithelial lymphocytes and subepithelial lymphocytes and plasma cells,[26,27] did not show any more inflammation than that observed in normal bulls.[29] Genital infection with *T. foetus* is persistent since *T. foetus* was consistently isolated by culture of smegma samples up to the time of slaughter at 4-18 months post-infection in 9 experimentally infected bulls (2-7 years old) and at 3-12 months from initial diagnosis in 15 naturally infected bulls (3-7 years old).[26] In addition, most bulls (8/9; 3-6 years old) challenged with different concentrations of *T. foetus* (10^2-10^6) became persistently infected until collection was terminated after 5 to 14 months.[30] In this same study, all bulls (5/5; 2-7 years old) challenged with 2.5 x10^6 *T. foetus* were also infected until slaughter 2 months later.[30] However, young bulls seem to be more resistant to *T. foetus* infection or eliminate it faster since, in one study, 16/18 young bulls (1-2 years old) were refractory to infection and the remaining 2 bulls were temporary carriers for less than 4 months.[30] Normal modifications in the preputial and penile epithelium of aged bulls (>5 years old), including more mucosal folds and deeper crypts, may provide a reduced oxygen tension niche that increases susceptibility to infections with the anaerobic *T. foetus*.[30]

Infection of bulls with *T. foetus* is considered to have limited or no effect on male fertility because *T. foetus* does not inhabit the male urethra[26,27] and its presence in semen is improbable.[26] Although bovine sperm cells (1 x10^6) were damaged or killed by exposure *in vitro* (30 min-6 h at 37°C) to *T. foetus* (1 x10^6; other concentrations of parasites or sperm were not reported),[31] this cytotoxic effect of *T. foetus* would be greatly reduced (i.e., diluted) in natural infections in which trichomonads are rarely found in semen[26] and bulls usually ejaculate 6 x10^9 sperms per coitus. Genital antibody responses to *T. foetus* infection in bulls, even when measurable, were incapable of eliminating genital infection since surface antigen specific IgG1, IgA and IgM antibodies in smegma of bulls naturally infected with *T. foetus*, measured by ELISA at one time point, coexisted with *T. foetus* positive smegma cultures.[27] Thus,

T. foetus appears not to be killed or induces pathogen specific inflammation in bulls since it persistently inhabits the lower genital tract for long periods of time with no clinical signs.

Campylobacter fetus subsp. *venerealis*. As described for *T. foetus*, *C. fetus* subsp. *venerealis* also persistently and asymptomatically colonizes the epithelium of the prepuce and penis of bulls. In one study, *C. fetus* subsp. *venerealis* was identified by immunofluorescence on impression smears and epithelial scrapings in the prepuce and penis of 6/6 bulls (5.5-12 years old), 5 of them as carriers as shown by culture of smegma samples, but only 1/6 bulls were colonized in the distal urethra.[32] In addition, immunofluorescent studies on tissue sections showed that *C. fetus* subsp. *venerealis* colonized the preputial and penile epithelial surface of the lumen and the crypts but never the deep epithelium or subepithelium.[32]

Infection with *C. fetus* subsp. *venerealis* in bulls was not associated with any clinical signs, altered semen quality,[33,34] or gross genital abnormalities.[35] Microscopically, two studies, each with 6 *C. fetus* subsp. *venerealis*-infected bulls, agreed that the subepithelium of the prepuce and penis were infiltrated by lymphocytes and plasma cells, but this inflammatory response was non pathognomonic for *C. fetus* subsp. *venerealis.*[32,35] In agreement, the number of total non-antigen specific plasma cells in the subepithelium of the prepuce were similar between 4 bulls infected with *C. fetus* subsp. *venerealis* and 79 control bulls.[29]

Genital infection with *C. fetus* subsp. *venerealis* among mature bulls (>4 years old) appeared to be persistent, as determined by consecutive *C. fetus* subsp. *venerealis* positive smegma cultures. After intrapreputial challenge with *C. fetus* subsp. *venerealis* (4.8 $\times 10^9$), 4/4 old (66-74 months old) and 3/4 young (41-49 months old) bulls were infected until slaughter, at 9-10 and 16-18 weeks after challenge, respectively, while the remaining young bull was infected for only 4 weeks.[35] In agreement, 4/6 bulls (5 years old) intrapreputially challenged with *C. fetus* subsp. *venerealis* (3 times with 2.5-4.5 $\times 10^8$ organisms) were infected for at least 4 months in an experiment terminated at 4 months.36 It is uncertain whether the genital infection with *C. fetus* subsp. *venerealis* is influenced by the age of bulls. In one study, the incidence of *C. fetus* subsp. *venerealis* in semen was higher in old (> 6 years old) (65/139) than in young (< 6 years old) (4/233) bulls (with a variable number of cultures per bull)[37] but, in another, the preputial and penile mucosa was microscopically similar between old and young bulls, both similarly infected with *C. fetus* subsp. *venerealis.*[35]

Antibody response to *C. fetus* subsp. *venerealis* in bulls, even when measurable, did not clear the infection. Genital agglutinating antibodies specific to *C. fetus* subsp. *venerealis* coexisted with *C. fetus* subsp. *venerealis* positive cultures in smegma of two naturally infected bulls.[19] On the other hand, in other studies, specific *C. fetus* subsp. *venerealis* agglutinins were absent in smegma of 6 naturally infected bulls[32] and the levels of specific agglutinins were similar between 8 experimentally infected bulls (7 of them carriers as shown by smegma culture) and 2 uninfected bulls.[35] Likewise, no systemic immune response was detected by tube agglutination tests (O somatic-cell wall and superficial K heat-labile antigens) in 4 bulls infected with *C. fetus* subsp. *venerealis* for at least 4 months after challenge.[36] These studies demonstrate that *C. fetus* subsp. *venerealis* could inhabit the lower genital tract of bulls for at least a few months and in the absence of an effective specific antibody response.

Cows-pregnancy loss due to STDs

Tritrichomonas foetus. Infection with *T. foetus* in bovine females, in contrast to bulls, provoked genital inflammation including vaginitis, cervicitis and endometritis[38-43] and, in pregnant cows, fetal death in the first trimester of gestation[39] or later.[44] In one experimental study, this genital inflammation and pregnancy loss occurred after 7 weeks of infection.[39] Moreover, infection with *T. foetus* in female cattle does induce a measurable antigen specific antibody response in the vagina. Intra-vaginal inoculation of non-pregnant heifers with *T. foetus* (7 $\times 10^6$) induced whole *T. foetus* cell specific IgG1 and IgA antibodies in vaginal secretions at 7-9 weeks[45] and IgA persisted longer for 24 weeks after infection or until the time of genital clearance.[46] Lower intra-vaginal doses of *T. foetus* (1 $\times 10^6$) similarly induced vaginal IgG1 and IgA antibodies specific to TF1.17 antigens at 5-10 weeks[38,41,42] and to whole *T. foetus* cell antigens at 5-8 weeks.[43,47] Also differing from bulls, the genital infection in heifers was limited to 13-28 weeks (vaginal

challenge of 7 x10^6 *T. foetus*)[45] and many of the infected heifers cleared infection by 6-10 weeks in studies terminated at 8-12 weeks after challenge (vaginal challenge of 1 x10^6 *T. foetus*).[38,40-43,47] The above studies suggested that the immunity acquired by infection with *T. foetus* in bovine females usually results in a brief genital infection compared to bulls; however, it cannot prevent reproductive failures.

Such non-protective natural genital antibody response to *T. foetus* might be due to parasite factors capable of affecting local immunity by masking antigens or digesting proteins involved in innate and acquired immunity. Virulent factors of *T. foetus* include secreted extracellular cysteine proteinases[48] which, *in vitro*, digest fibrinogen, fibronectin, albumin, lactoferrin,[49] the third component of the complement (C3)[50] and IgG1 and IgG2 antibodies.[49] Since these cysteine proteinases preferentially cleave IgG2^a allotypes rather than IgG2^b,[51] long-term *T. foetus* infection with low levels of local IgG2 antibodies may be due to a host genetic predominance of the susceptible IgG2^a allotype. Otherwise, *T. foetus* non-specifically binds bovine IgG2 and to a lesser extent IgG1 isotypes[52] and this mechanism may possibly shield the parasite from antibody recognition of masked antigens in the effector stage of the immune response.

Campylobacter fetus subsp. *venerealis.* Natural infection with *C. fetus* subsp. *venerealis* in cows is associated with reproductive failure, including irregular estrus, transient infertility and, in pregnant cows, embryonic-fetal death.[25,34, 53-56] Moreover, experimental intrauterine and cervico-vaginal infection with *C. fetus* subsp. *venerealis* in female cattle provoked different grades of genital inflammation, including vaginitis, cervicitis, endometritis, and salpingitis.[57] Genital infection with *C. fetus* subsp. *venerealis* in female cattle, differing from bulls, does induce a detectable antibody response in the lower genital tract (i.e., vagina). In one study, using indirect fluorescence antibody assays, intra-vaginal inoculation of non-pregnant heifers with *C. fetus* subsp. *venerealis* (1 x10^4) induced antigen specific transient IgM followed by persistent IgA and IgG antibody responses in cervico-vaginal secretions and a IgG1 response in uterine secretions.[58] Likewise, higher intra-vaginal doses of *C. fetus* subsp. *venerealis* (2-4 x10^6 organisms) induced specific agglutinating and immobilizing antibodies in cervico-vaginal secretions of heifers.[59] In addition, a vaginal specific IgA antibody response was detected by ELISA after abortion in 7 heifers naturally infected with *C. fetus* subsp. *venerealis.*[55] Genital infection with *C. fetus* subsp. *venerealis* in female cattle, contrasting with bulls, was limited to weeks or months in the uterus and oviducts where there was a predominance of IgG antibodies[53,57,58] but persisted longer, for 6-18 months or even 24 months, in the vagina where there was a predominance of IgA antibodies.[53,58,59] These carrier cows could maintain the infection in vaginal mucus for longer periods, perhaps from one breeding season to another. However, some cows (up to 5 per cent), may be resistant to infection after repeated exposure to carrier bulls or the deposition of large numbers of viable organisms into the reproductive tract.[60]

How to diagnose of bovine STDs?

Culture and polymerase chain reaction for detecting STDs in genital secretions

Vaginal and preputial secretions are usually sampled by introducing an insemination/infusion pipette inside the vaginal fornix or preputial cavity and moving it back and forth in short strokes while aspirating the secretions.[61] Phosphate buffered saline solution (PBS) may be added to wash the cavity and recover higher amounts of samples, although it may dilute the sample. Alternatively, preputial secretions can be taken by scraping the cavity using a metal brush with no significant differences in culture sensitivity (Se) compared with the pipette.[62] Diagnosis of trichomoniasis in genital secretions, and campylobacteriosis although much less commonly utilized, are mostly based on culture and/or polymerase chain reaction (PCR), as described below.

Culture of Tritrichomonas foetus. The most routinely employed diagnostic test for bovine trichomoniasis is culturing preputial smegma or vaginal secretions into selected media, such as Diamond's,[63] Plastridge,[64] In Pouch TF® (Biomed Diagnostics, San Jose, CA),[65] or liver infusion broth.[66] Samples are incubated at 37°C (98.6°F) and examined on days 1, 3, 5 and 7 after sampling by placing a

drop on a glass slide and observed at 40-100x magnification using light microscopy. Samples are considered positive when living trichomonads with size, shape and a wave-like, rapid and irregular jerky movement of the protozoan body compatible with *T. foetus* were observed.[67] Although diagnostic Se of In Pouch TF® and Diamond's for detecting *T. foetus* in bulls differ among studies (88%-98.4%;[68] 81.6%-93.2%[63]), differences between the culture media sensitivities were not significant.[69,70]

Polymerase chain reaction of Tritrichomonas foetus. Diagnosis of *T. foetus* in smegma of bulls by PCR is theoretically as sensitive and specific as cultures because it relies on the amplification of DNA from the organism and not on the successful culture of live organisms. Polymerase chain reacthon methods targeting the 5.8S rRNA gene and the flanking internal transcribed spacer (ITS1 and ITS2) regions of *T. foetus* has found wide acceptance and has been applied to detected *T. foetus* from cultured isolates, cervico-vaginal mucus, and tissues from female genitalia; and, *in vitro*, it differentiated *T. foetus* from other morphologically similar trichomonads.[71,72] This PCR includes a set of primers, TFR1 and TFR2 complementary to conserved sequences of the 3'-end of the 18S subunit rRNA gene and the 5'-end of the 28S subunit rRNA gene, that define the *Trichomonadidae* family by yielding a product of 372-bp. Other set of primers, TFR3 complementary to the 5' end of the 28S rRNA gene and TFR4 to the 18S rRNA gene and ITS1, specifically target *T. foetus* yielding a product of 347 bp.[71,72] The *in vitro* biological Se of TFR3/4 was approximately 90% and the specificity (Sp) of 98%,[73] detecting as little as 0.03 pg of purified *T. foetus* DNA[72] or approximately 2 organisms/mL of sheath-wash samples under laboratory conditions.[73] Other PCR-based tests include a loop mediated isothermal amplification targeting the 5.8S rDNA subunit designed for the specific identification of *T. foetus* in smegma that was as sensitive and specific as PCR amplification with TFR3 and TFR4 primers.[74] A *T. foetus*-specific 5' Taq nuclease assay using a 3' minor groove binder-DNA probe (TaqMan MGB) targeting conserved regions of the ITS-1 is also available for detecting *T. foetus* in genital secretions.[75] This probe-based real time PCR assay was more sensitive than InPouch TF® culture and the conventional TFR3-TFR4 PCR assay detecting *T. foetus* in spiked smegma or mucus specimens[75] or in pooled protozoal cultures of preputial scraping samples.[76,77] Thus, although variability in methodologies used by the laboratories affects the Se and Sp of qPCR, this test seems to be rapid, quantitative, reliable and accurate in detecting *T. foetus*[77] and, in addition it has been recently implemented by California Department of Food and Agriculture (CDFA) for diagnosis of bovine trichomoniasis. As a general rule, genital secretions sent to the laboratory for diagnosing STDs should be transported at room temperature for culture and at 4°C for PCR.[61]

A new concern in the diagnosis of bovine trichomoniasis-the false trichomonads

Culture isolation of trichomonads from preputial secretions of bulls, others than *T. foetus,* infers a risk of false Sp in the diagnosis of *T. foetus* by culture. Trichomonads morphologically similarly under low microscopic observation to *T. foetus* were isolated from cultured preputial secretions of virgin bulls from widely dispersed geographical areas, including the western of USA,[78,79] Canada,[80] and Argentina.[81] Since the virgin bulls had not been used for breeding, these non-sexually transmitted trichomonads were generalized as "non-*T. foetus* trichomonads". Transmission and scanning electron microscopy identified *Tetratrichomonas spp.* in non-*T. foetus* trichomonads isolates.[81] Fortunately, amplified sequences of the 5.8S rRNA gene and ITSRs and restriction fragment length polymorphism (RFLP) tests in lieu of DNA sequencing differentiated *T. foetus* from *Pentatrichomonas hominis* (*P. hominis*) and *Tetratrichomonas spp.* recovered from the bovine preputial cavity.[78,82]

Confronted with this new problem, the persistence and pathogenicity of tetratrichomonads and *P. hominis* has been investigated in bulls and cows. These studies showed tetratrichomonads are only detected intermittently in the female genital tract and produced no histological lesions.[43,83] Likewise, *Tetratrichomonas spp.* did not survive in experimentally infected bulls[83] or were only detected for a short period in naturally infected bulls.[80] Trichomonads including *Tetratrichomonas spp* are commensal species in the bovine intestinal tract.[84-86] Consequently, the intermittently finding of *Tetratrichomonas spp.* in genital secretions but frequent isolation in feces suggests a fecal-genital route of contamination. This fecal-genital route of contamination is supported by the fact that tetratrichomonads may appear in

the lower genital tract in bulls due to homosexual behavior.[78-82] Thus, "non-*T. foetus* trichomonads" including *Tetratrichomonas spp.* from the intestinal tract could sporadically contaminate the genital tract as a consequence of defecation and physical contact among cows and bulls, inhabiting temporarily the genital tract and confusing the diagnosis of trichomoniasis.[79,81,83,87]

Combination of culture and PCR for a faster and reliable diagnosis of bovine trichomoniasis

Diagnosis of *T. foetus* by culture only is a limited approach because Se varied from 84% to 96% under experimental conditions[63,88-90] or even lower under field suboptimal conditions[73] and it lacks Sp since *Tetratrichomonads spp.* can be isolated from cultured preputial smegma.[67,80,81,87] As a result, the US AI industry prescribes for bovine trichomoniasis a rigorous protocol of six weekly *T. foetus* negative cultures for bulls older than 365 days of age.[91] This diagnostic routine of six weekly cultures for diagnosing *T. foetus* in bulls has proven to be highly effective in controlling disease, with a Se of 86.7% and Sp of 97.5%.[61] However, the use of culture and PCR, individually on the same sample split in two, one for culture and one for PCR, for three consecutive weeks (Se 87.5%, Sp 95.6%) appeared to be similar to this standard of six weekly cultures.[61] The same study proved that a single culture or PCR seems to be equally sensitive detecting *T. foetus* (Se of 67.8% and 65.9%, respectively) with Sp greater than 90%.[61] The similar performance of culture and PCR agrees with *in vitro* studies where PCR made from 5-day cultures of male genital secretions agreed 92.9% with culture.[73] Combination of culture and PCR for *T. foetus* diagnostics offers high Se and improved Sp and may require less time and perhaps less cost in the surveillance of AI bulls and beef bulls prior to the breeding season.

Culture of C. fetus subsp. *venerealis.* For isolating *C. fetus* subsp. *venerealis*, samples obtained from preputial or vaginal secretions, or fetal stomach contents, lungs, liver and placentome, for abortion investigations, are transported to the laboratory in transport enrichment medium (TEM) at room temperature. In the laboratory, samples are cultured in selective culture media, such as 5% sheep blood agar, Skirrow's agar[92] or Clark's selective agar in microaerophilic conditions containing 5–7% oxygen, 5–15% carbon dioxide and 65–90% nitrogen and incubated at 37 ±2°C for 48h. Passive filtration of fresh preputial scrapings onto blood agar yielded higher recovery rates of *C. fetus* subsp. *venerealis* than direct plating.[93] Subspecies of *Campylobacter* are differentiated biochemically by tolerance to 1% glycine and production of hydrogen sulfide through the utilization of cysteine in which presumptive *C. fetus* subsp. *fetus* colonies but not *C. fetus* subsp. *venerealis*, will growth with 1% glycine or with NaCl and cysteine.[94] However, culture of *C. fetus* subsp. *venerealis* has severe practical limitations and is not widely used as diagnostic test because it has low Se and is laborious and time-consuming. Also, some strains of *C. fetus* subsp. *venerealis* are sensitive to polymyxin B (common in both TEM and selective media), *C. fetus venerealis* biovar "*intermedius*" can tolerate higher concentrations of glycine, and commensal bacteria easily overgrow into these culture media. In addition, transport of samples containing *C. fetus* subsp. *venerealis* is critical as excessively cool or hot temperatures or transit times longer than 24 hours will make recovery of *C. fetus* subsp. *venerealis* very unlikely.[95]

PCR for C. fetus subsp. *venerealis.* Diagnosis of campylobacteriosis by PCR-based test should not only identify *C. fetus* subsp. *venerealis* but also differentiate it from *C. fetus* subsp. *fetus*. Misidentification of *C. fetus* subsp. *venerealis* as *C. fetus* subsp. *fetus* results into the spread of *C. fetus* subsp. *venerealis* into cattle populations and *C. fetus* subsp. *fetus* as *C. fetus* subsp. *venerealis* results in economic losses.[96] *C. fetus* subsp. *venerealis* has high sequence identity (92%) with *C. fetus* subsp. *fetus* but possess unique elements that include a pathogenicity island (30-kb element) for encoding genes phylogenetically related to the VirB–VirD4 operon for bacterial type IV secretion system and mobility genes such as phage integrase and insertion sequence (IS) transposase.[97-100] These genomic particularities of *C. fetus* subsp. *venerealis* have been useful for designing several PCR primer sequences for identification and differentiation of *C. fetus* subsp. *venerealis*.[92,96,101] One of the most reliable PCR and quantitative PCR test includes one primer set (MG3F/MG4R) that amplifies a 750-960 base pair fragment of the *C. fetus* carbon starvation protein gene, found in both *C. fetus* subspecies, and other primer set (VenSF/VenSR) that amplifies a 142 bp fragment of the parA gene, exclusive of *C. fetus* subsp. *venerealis*.[102] A SYBR Green qPCR based on VenSF/VenSR primer set was likewise optimized for

detecting *C. fetus* subsp. *venerealis* directly in preputial samples.[92] A multiplex PCR assay using a set of primer to amplify a *C. fetus*-specific 764-bp sequence and other set of primers (nC1165g4F/nC1165g4R) to amplify a 233-bp sequence that is only present in *C. fetus* subsp. *venerealis* also detected and differentiated between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* in samples of abomasal liquid of aborted bovine fetuses without any pre-enrichment step.[94] Likewise, two real time SYBR Green PCR assays for the detection and discrimination of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* showed high Se and Sp for *C. fetus* (CampF4/R4; 100% and 99.6%, respectively) and *C. fetus venerealis* (CampF7/R7; 98.7% and 99.8%, respectively) on 1071 bacterial isolates.[101] However, molecular analysis by amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST) may be still recommended to identify *C. fetus* subspecies isolates as other real-time PCR assay targeting gene *nahE* showed 100% Se and Sp for *C. fetus* species but the subspecies *venerealis* specific real-time PCR (ISC*fe1*) failed due to sequence variation of the target insertion sequence.[96] Thus, diagnosis of campylobacteriosis still needs further investigation to be routinely used for control and eradication because culture is unpractical and PCR is not widely standardized. The recent complete sequencing genome of *C. fetus* subsp. *venerealis*[103] will lead to new improved molecular diagnostic tools (e.g. real-time PCR, PCR) for identification of *C. fetus* subsp. *venerealis.*

Control of STDs by testing and vaccination

There is no effective legal treatment for *T. foetus* since nitro-imidazole drugs showed some efficacy but they are not allowed in cattle. In addition, antibiotic treatments for *C. fetus* subsp. *venerealis* are impractical and with doubtful efficacy. Thus, control of bovine STDs involves diagnostic testing of animals to identify positive animals and depending on the local laws, culling of infected animals. For instance the control program disposed by the CDFA in partnership with the livestock industry declares that: bulls infected *T. foetus* should be permanently quarantined until they go to slaughter and herdmate bulls should be quarantined until one negative real-time PCR test or three consecutive negative trichomoniasis culture tests. Importantly, both STDs need to be reported to local authorities. The veterinarian approved for trichomoniasis sampling that collects the samples is required to report all positive and negative test results to CDFA within two days of the final laboratory reading date, and negative tests must be reported within 30 days. Campylobacteriosis belongs to list B of the notifiable disease of the International Office of Epizootics (OIE) and animals or animal products must be certified as *C. fetus* subsp. *venerealis*-free for international trade.[104] Hence, the AI industry demands rigorous testing standards and protocols for assuring semen free of pathogens, as bulls are the epidemiologic natural reservoir and a major factor in maintaining and transmitting STDs. For controlling *C. fetus* subsp. *venerealis* and *T. foetus* infections, for example in the USA, the Certified Semen Services, Inc. (a subsidiary of the National Association of Animal Breeders) requires a series of weekly negative cultures of smegma from each bull with an age-dependent regime (1 test for bulls <6 month old, 3 tests for bulls 6 month-1 year old, 6 tests for bulls >1 year old). Furthermore, the AI industry routinely adds antibiotics to the semen in the freezing process although cases of bacterial antibiotic resistance have been reported for *C. fetus* subsp. *venerealis.*[105] Thus, diagnosis of *C. fetus* subsp. *venerealis* and *T. foetus*, isolation of infected animals and notification to authorities are foundational steps to achieve success programs of control and eradication of STDs.

Systemic vaccination with *T. foetus* and *C. fetus* subsp. *venerealis* in cattle has been associated with prevention and cure of genital infection by inducing measurable systemic IgG antibodies, which may translocate to genital secretions. We continue to review important studies investigating vaccines against STDs in cows and bulls.

Vaccines against Tritrichomonas foetus. Several studies have been shown that systemic immunization in bulls with antigens of *T. foetus*[106,107] prevents or clears genital infections, based on culture of smegma samples. In one study, whole *T. foetus* cell antigens in a mineral oil adjuvant were systemically given 3 times at monthly intervals to age susceptible bulls (>4 years old).[106] Then, bulls were challenged with *T. foetus* by mating with infected cows or by intrapreputial inoculation at 1 and 6 months after the third vaccine dose and considered free of infection after 5 consecutive weekly negative

smegma cultures and persistently infected after 9 consecutive *T. foetus* positive smegma cultures. The results showed that whole *T. foetus* cell antigens prevented or shortened infection in 37/48 vaccinated bulls whereas merely 18/38 control unvaccinated challenged bulls remained free of infection or infected for a short time.[106] The same study showed that therapeutic immunization with this whole *T. foetus* cell antigen cleared infection in 11/16 vaccinated bulls while only 1/8 untreated bulls eliminated infection.[106] This whole *T. foetus* cell vaccine was most effective in bulls less that 5.5 years of age[106] because young bulls may be more difficult to infect since their preputial crypts less deep would not favor microaerophilic/anaerobic microbes. Studies using other types of *T. foetus* antigens showed also some efficacy. In one study, membrane preparation (500 µg/dose) or purified membrane glycoprotein (160 µg/dose) antigens of *T. foetus*, both in a mineral oil adjuvant, were systemically given 3 times at monthly intervals to bulls with pre-existing genital *T. foetus* infection.[107] The results showed that 3/4 bulls vaccinated with membrane and 3/4 bulls vaccinated with membrane glycoprotein eliminated infection at week 2 after the second vaccine dose while the 2 remaining vaccinated bulls and 8/8 infected unvaccinated control bulls were infected for 2 months after the last dose.[107] The 3 bulls vaccinated with *T. foetus* membrane antigen that cleared infection and an additional 3 non-infected, non-vaccinated control bulls were subsequently challenged with *T. foetus* and none of the vaccinated bulls became infected while 2/3 control bulls acquired infection.[107] In a more recent study, systemic immunization of bulls with subcutaneous inoculation of 2 mL of a commercial vaccine containing whole-cell killed *T. foetus* in oil adjuvant prevented trichomonad colonization of the preputial and penile mucosa of 4/4 bulls vaccinated and challenged with *T. foetus.*[108] The vaccinated-challenged bulls had systemic and preputial *T. foetus* LPG/protein *T. foetus* antigen specific IgG1 and IgG2 and slight preputial IgE and IgA antibody responses that determined the resistance to trichomonad genital colonization.[108] These high and persistent levels of serum IgG antibodies in vaccinated bulls together with additional genital IgG antibody responses after the systemic vaccine doses imply that vaccine-induced systemic antibodies were significant contributors to the luminal IgG antibody response. The peak of preputial IgE antibody in vaccinated bulls before challenge, at the same time as the IgG1 and IgG2 antibodies, may aid translocation of serum IgG into smegma. Immunoglobulin E antibodies would cross-link *T. foetus* antigens on mast cell receptors to activate and release mediators increasing endothelial and epithelial permeability. This would facilitate systemic IgG antibody translocation from the bloodstream and across the genital epithelium into secretions as was proposed in infected heifers.[40] A significant genital specific IgA antibody response was detected before challenge in vaccinated bulls that could contribute to the resistance to trichomonad colonization.[108] These vaccinated-challenged bulls also had increased epithelial antigen presenting cells (MHC II$^+$ and CD205$^+$), CD3$^+$ and CD8$^+$T lymphocytes, and subepithelial B cells, IgG1 and IgA containing cells in the prepuce that may induce local responses as a part of cell-mediated immune response that prevent genital colonization.[108]

Systemic vaccination of non-pregnant heifers with an immunoaffinity purified lipophosphoglycan (LPG)/protein complex (TF1.17) antigen of *T. foetus* induces specific IgG1 and IgA antibodies in vaginal and uterine secretions that coincide with clearance of experimental genital infections, usually before 7 weeks.[38,41,42,47] Genital clearance before 7 weeks in female cattle likely prevents reproductive failures since inflammation and pregnancy loss did not occur until after 7 weeks of infection in an earlier study.[39] Likewise, systemic vaccination with whole cell[109-115] or membrane antigens[114] in cows mated with *T. foetus* infected bulls and experimentally infected shortened genital infection and improved calving rates compared with unvaccinated cows. In female cattle, booster vaccination with *T. foetus* antigens in the nasal[41] or vaginal mucosa[42] similarly elicited genital immunity. In these studies, virgin heifers were systemically vaccinated twice with immune purified TF1.17 antigen (100 µg), boosted with formalized whole *T. foetus* cells (10^8) given intranasally (6 heifers)[41] or intravaginally (9 heifers),[42] and then intravaginally challenged with *T. foetus* (10^6) 2-3 weeks later (all vaccine doses in Quil A and given 3 weeks apart). The results showed that systemic priming with vaginal or nasal boosting similarly induced a shortened infection and antigen specific vaginal IgA (3-10 weeks after challenge) and uterine IgA and IgG1 antibodies (10 weeks after challenge).[41,42] The same vaccination/challenge scheme with the booster given systemically in 6[41] and 10[42] heifers also induced shortened infection but mainly vaginal and uterine

IgG1 antibodies. Other specific *T. foetus* antigens, such as Tf190, also resulted in a lower number of infected heifers after experimental inoculation and elicited systemic IgG1 and IgG2 when injected subcutaneously and genital IgA when intra-nasally applied.[116] Thus, vaccination against trichomoniasis offers some advantages but better antigens and further research evaluating them in controlled and field conditions may be needed as a recent meta-analysis of the efficacy of whole-cell killed *T. foetus* vaccines in beef cattle showed that the impact of vaccination is still limited/low on infection and abortion risk in heifers and on infections in bulls.[117]

Vaccines against Campylobacter fetus subsp. venerealis. Systemic vaccination of bulls with *C. fetus* subsp. *venerealis* antigens has also resulted in genital protection.[36,118,119] In one study, whole *C. fetus* subsp. *venerealis* cell antigens (~40 mg dry matter weight) in mineral oil adjuvant were systemically given to bulls (14-18 months old) in twice a 2 month intervals and then annually.[119] The bulls were intrapreputially challenged with *C. fetus* subsp. *venerealis* every 6 months for a total of 5 times and considered free of infection after 4 consecutive weekly negative smegma cultures.[119] The results showed that 16/16 vaccinated bulls were free of infection but, of 17 control unvaccinated challenged bulls, 13 were infected for 3 or more months and only 2 were free of infection and 2 infected for less than 2 weeks.[119] The ages at which bulls became infected ranged from 2-6 years.[119] Likewise, in another study, a dual vaccine containing *C. fetus* subsp. *intermedius* and *C. fetus* subsp. *venerealis* antigens (20 mg of each) was given to bulls (20-34 months old) using the same vaccination/challenge scheme.[120] The results showed that 5/5 bulls challenged with *C. fetus* subsp. *intermedius* and 5/5 bulls challenged with *C. fetus* subsp. *venerealis* remained free of infection while 9/10 challenged unvaccinated control bulls were infected for at least 5 weeks after challenge.[120] The ages at which bulls became infected ranged from 3.5-5.5 years.[120] Therapeutic immunization against *C. fetus* subsp. *venerealis* infection in bulls has also demonstrated some effectiveness. In one study, a commercial whole *C. fetus* subsp. *venerealis* cell antigen vaccine (~40 mg dry matter weight) was systemically given in twice a month intervals to bulls already infected (5 years old).[36] Infection status was determined by smegma culture for 8 weeks after primary vaccination and by culture of genital secretions of a virgin heifer after natural service by the tested bull. The results showed that 6/6 infected vaccinated bulls cleared infection while 4/4 infected and unvaccinated bulls remained infected for 4 months, at which time they were vaccinated and 2 of them cleared the infection.[36] In another study in which infection status was evaluated by smegma culture and immunofluorescence tests, a vaccine containing whole *C. fetus subsp. intestinalis* cell antigens in incomplete Freund's adjuvant (concentration unreported) was systemically given once to 288 bulls serving in a *C. fetus* subsp. *venerealis*-infected area and none of the bulls acquired infection.[118] Moreover, this same vaccine given twice cleared infection by 42 days after the second dose in 41/41 bulls already infected with *C. fetus* subsp. *venerealis* and exposed to infected females and, prevented re infections in 8/8 bulls that previously cleared infection by vaccination and were intrapreputially challenged 2-8 times with *C. fetus* subsp. *venerealis* (unreported doses).[118] However, in this study, results from control (unvaccinated) bulls were not reported, antigenic similarity between the infecting *C. fetus* subsp. *venerealis* and the immunogen *C. fetus* subsp. *intestinalis* was undetermined, and mating in an endemic area may not have been a sufficient challenge. Regarding immunity induced by *C. fetus* subsp. *venerealis* antigens, systemic vaccination with whole *C. fetus* subsp. *venerealis* antigen, given twice at a monthly interval, induced higher titers of serum agglutinins to heat-labile K antigens in 10 vaccinated bulls (agglutination range 500-1280, with peak at 6 weeks after the first vaccine dose) than in 2 unvaccinated bulls (agglutination range 60-80).[36] Likewise, systemic vaccination with whole *C. fetus* subsp. *venerealis* cell antigen, given twice at a 2 month interval and annually, induced higher titers of serum agglutinins in 12/17 vaccinated bulls (agglutination range 40-40,960, with peak at 3 weeks after the second vaccine dose) than in 17 unvaccinated control bulls (agglutination range usually less than 20 and the highest only 160).[119]

Systemic immunization of cows with bacterins of *C. fetus* subsp. *venerealis* has been associated with prevention and even cure of infection. Systemic vaccination with *C. fetus* subsp. *venerealis* and biotype *intermedius* (20 mg dry weight of each one) in mineral oil adjuvant vaccine protected cows against genital experimental infection with either organism.[121] Heifers vaccinated with bacterin

containing K antigen were resistant to experimental infection with *C. fetus* subsp. *fetus.*[122] Moreover, systemic immunization with killed *C. fetus* cells in incomplete Freund's adjuvant also cured infection in 6/8 cows previously infected with *C. fetus* subsp. *venerealis.*[123] This protective and curative effect of systemic vaccination has been largely associated with the induced antibody response as described in bulls. Systemic vaccines with *C. fetus* bacterins in oil adjuvant stimulated high level of systemic IgG1 and IgG2, and genital IgG1 and IgG2.[59,124] However, some vaccine failures have been reported too. Two commercial vaccines containing *C. fetus* subsp. *venerealis* applied subcutaneously in female cattle naturally challenged by serving with infected bull during 60 days did not protect them against infection of *C. fetus subsp. venerealis.*[125] In spite of the vaccination, vaccinated and control groups showed a high percentage of infected heifers and both groups showed a poor reproductive performance. Likewise therapeutic vaccination failed in a 4 year old bull infected with *C. fetus* subsp. *venerealis*, since he remained infected after systemic immunization with 2 different commercial whole cell vaccines, given twice and 3 times, respectively.[126] However, after this bull was cured by antibiotic treatment, he remained free of infection to an intrapreputial challenge.[126]

Failures in vaccines against campylobacteriosis may rely on two factors: antigenic differences between regional and standard strains and/or insufficient content of dry weight *C. fetus* cells. Variation on the surface antigens of *C. fetus* subsp. *venerealis* may impede recognition by the immune system and the consequent limited immune response to infection. Isolates of *C. fetus* subsp. *venerealis* from smegma of 3/4 relatively young (41-49 months old) and 3/4 older (66-74 months old) infected bulls modified their superficial antigens, in that rabbit antiserum specific to whole heat labile surface *C. fetus* subsp. *venerealis* antigens showed decreased agglutination titers to individual antigens of subsequent isolates compared with the infecting strain.[35] Superficial antigenic variation was also evidenced by agglutination tests in *C. fetus* subsp. *venerealis* isolates from cervico-vaginal mucus of 2 heifers over several months of infection.[127] In this study, the isolates, sampled throughout infection, reacted with rabbit antiserum of various specificities and the specificity of the cervico-vaginal agglutinating antibodies of the heifers varied during infection.[127] As alternative mechanisms of evasion, *C. fetus* subsp. *venerealis* may bind bovine IgA specific antibodies, thus escaping the complement and phagocytosis-mediating properties of IgG[128] and bacterial surface glycoproteins, in the absence of specific antibodies, inhibit ingestion by macrophages.[129] Hence, microbial antigenic variation and capacity for blocking inflammatory-immune effectors (e.g., complement, IgG, macrophages) may promote persistence of *C. fetus* subsp. *venerealis* and failure of vaccines with international strains only. In addition, many commercial vaccines include *C. fetus* subsp. *venerealis* only and lack *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* biotype *intermedius* although they were also associated with reproductive problems such as infertility, lowered pregnancy rates, and abortion in cattle.[2,3,56,94,130] Dry weight cells may also determine the effectiveness of the vaccine as experimental vaccines gave a good protection against genital infection only when they contained at least 40 mg of dry weight per dose in oil adjuvant.[121] However, the main limitation about vaccination against campylobacteriosis in cows and bulls is the lack of more updated reports and the testing of the current as well as new vaccines.

There is little information about the effectiveness of vaccines containing both *C. fetus* subsp. *venerealis* and *T. foetus* antigens. One study evaluated systemic vaccination with whole cells of *C. fetus* subsp. *venerealis* and *T. foetus* in cows mated for 90 days with bulls infected with *C. fetus* subsp. *venerealis* and *T. foetus* (from day 0 to day +90) plus an additional vaginal instillation of both pathogens at Day +39.[115] Vaccines were administrated subcutaneously at days -30 and +11 and into the vaginal submucosa at day -9 of the mating period. Vaccinated animals showed elevated systemic and vaginal IgG antibody response followed by shorter infections with both pathogens and improved pregnancy rate.[115] This vaccination scheme combining systemic subcutaneous and mucosal vaginal doses near and within breeding period could induce lasting immune response that will cover the critical risk period in a 2-3 month service program, from the last part of breeding season until one month after it, when most of pregnancy losses occur.[115]

Why IgG antibodies induced by systemic immunization may be the key for successful vaccines?

A protective role of serum IgG antibodies specific to *T. foetus* and *C. fetus* subsp. *venerealis* has been reported from *in vitro* and *in vivo* studies. Bovine whole *T. foetus* cell antigen specific serum inhibited trichomonad adherence to bovine vaginal epithelial cells and immobilized and agglutinated *T. foetus.*[131] In this same study, the relevance of IgG1 was evidenced since whole *T. foetus* cell antigen specific serum IgG1-enriched fractions, but not IgG2, inhibited adherence of *T. foetus* to bovine vaginal epithelial cells.[131] In addition, bovine immune serum or its IgG2 fraction in combination with complement enhanced the neutrophil-mediated destruction of *T. foetus*;[132] while bovine IgG antibody, but not IgA, stimulated the polymorphonuclear-mediated destruction of *C. fetus* subsp. *venerealis.*[128] Furthermore, bovine whole *T. foetus* cell antigen specific serum enhanced bovine complement-mediated destruction of *T. foetus.*[133,134] This enhanced complement-mediated *T. foetus* killing in presence of antibodies is likely via the classic pathway since bovine serum IgG1 and IgG2 immunoglobulins similarly fixed bovine complement via the classical pathway[135] and bovine serum IgG2 ($IgG2^b$ more than $IgG2^a$) induced complement mediated-lysis of anti guinea pig red blood cells via the same pathway.[136] However, it should be considered that complement alone (i.e., in the absence of antibodies) might kill *T. foetus* by the alternative pathway.[133,134] Besides, low quantity of antibodies may trigger complement-mediated *T. foetus* by the classic pathway and additionally, enhance complement-mediated *T. foetus* by the alterative pathway.

A protective role of IgG and IgA antibodies was also revealed in heifers vaccinated with TF1.17 antigen, in which antigen specific systemic IgG1 and vaginal and uterine IgG1 and IgA accelerated clearance of *T. foetus* infection before the time when lesions and reproductive failure occurs.[38,41,42,47] This vaccine-induced serum IgG1 antibody against 50-70 kDa and 150-200 kDa shed surface LPG *T. foetus* antigens is similar to previously reported reactivity of antibodies to TF1.17 and Tf190 LPG/protein adhesions.[137,138] Antibodies specific to surface LPG/protein antigens should protect against different strains since TF1.17 and Tf190 antigens are conserved among different isolates of *T. foetus.*[139,140] Thus, IgG antibody induced by vaccination by preventing adherence, activating complement and opsonizing pathogens for phagocytosis may define the outcome of STDs in the genital tract of bulls and cows.

Conclusion

Bovine STDs, trichomoniasis and campylobacteriosis, are still endemic mostly in beef cattle, causing silent economical detriment due to reproductive losses. Diagnosis of STDs is complicated and combinations of culture and PCRs consecutive times may be necessary for an adequate identification of diseased animals. Moreover, diagnosis of campylobacteriosis is especially difficult and currently not routinely used. Thus, it is very likely that STD prevalence and impact is underestimated, even more in areas with limited professional and economical resources. With no available treatment, identification and culling of infected animals is the only solution for the problem. Vaccines against STDs have been developed and few of them are on the market. However, the deficiencies in the diagnosis and still limited number of trials evaluating those vaccines impede to measure the real benefits of vaccinations. Sexually transmitted diseases have been known for decades but they continue to be the main reproductive diseases in cattle health. Collective actions among farmers, governments, industry and academia are needed for establishing and controlling regulations for further investigation in diagnosis, treatment and prophylaxis of STDs.

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Application of equine oocyte recovery and assisted reproductive techniques to clinical practice

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Assisted reproductive techniques (ART) are increasingly used to produce foals from mares with reproductive abnormalities, from stallions with limited semen availability or quality, or both.[1-5] While currently available in limited locations, it is possible to provide access to these services in private clinical practice. Most sophisticated reproductive practices can add a little equipment and, with proper preparation and practice, be able to recover oocytes useful for ART. These can then either be used for oocyte transfer or shipped to an ICSI (intracytoplasmic sperm injection) laboratory. Blastocysts resulting from ICSI can be transferred to recipient mares, frozen for future use, or shipped back to the referring veterinarian for transfer.

Oocyte development and follicular dynamics

A diverse population of follicles can exist on a single ovary at any given time during the estrous cycle, during transition, or at any time other than deep anestrus. Other than oocytes in pre-ovulatory follicles, oocytes in antral follicles are in the germinal vesicle (GV) stage. Germinal vesicle oocytes will differ in cytoplasmic maturity or atresia and thus vary in competency to resume meiosis. Oocytes are held in meiotic arrest by the follicle until luteinizing hormone (LH) activation, but the cytoplasm will mature over time. When removed from follicles oocytes will attempt to resume meiosis as soon as they are released from follicular inhibition.

Oocytes and associated cumulus masses in dominant follicles undergo significant changes as ovulation approaches. The granulosa cells surrounding the oocyte become the cumulus cells as part of the cumulus oocyte complex (COC). The cumulus mass expands in size as the cells secrete extra-cellular matrix, decreasing the firmness of the attachment of the oocyte to the follicular wall until the COC is released at ovulation. As a result of the LH surge the oocyte resumes meiosis characterized by germinal vesicle breakdown and formation of the meiotic spindle. The oocyte reaches metaphase II, characterized by extrusion of the first polar body, shortly before ovulation, and remaining in metaphase II until fertilization.

The right oocyte for the job

Oocyte requirements for ART vary between the individual techniques and the laboratories performing them. Oocyte transfer is most frequently performed with oocytes from dominant follicles stimulated (DSF) with an ovulation-inducing agent such as deslorelin or human chorionic gonadotropin (hCG). Oocyte recovery, transfer, and insemination of the recipient is based on the timing of the ovulation inducing agent maturation of the oocyte.[6] Based on laboratory preference, ICSI can either be performed with DSF oocytes with similar timing or with immature oocytes (IMM) in the GV stage recovered from small follicles and matured in the laboratory. The DSF oocyte has the advantage of having undergone natural selection and maturation, generally being the highest quality oocyte available from a given mare. It has the disadvantage of being temperature sensitive (the meiotic spindle can depolymerize from only mild and brief temperature fluctuations) as well as a limitation in numbers with only one or two dominant follicles per cycle. Immature oocytes can frequently be recovered in greater numbers, have decreased temperature sensitivity until meiosis resumes, and recovery is not dependent on the stage of estrus, simplifying scheduling. However, IMM oocytes have greater variation in competence.[7]

Oocyte culture basics

Basic oocyte culture should provide for temperature, pH maintenance, osmolality, electrolyte balance, and nutrition. Most oocyte culture systems are based on Medium 199 (M199), a classic tissue culture medium. There are two general formulations, Hanks' salts and Earle's salts. The Hanks' formulation, generally with 25mM HEPES, uses a phosphate buffer system to maintain pH in room

atmosphere, while Earle's includes a bicarbonate buffer system that maintains physiological pH in a 5% CO_2 atmosphere. Serum is added at 10% for pH stabilization, cellular nutrition, and growth factors. Pyruvate is added, being an important energy source for the oocyte and cumulus cells. Since phosphates and HEPES at a concentration above 10mM have been shown to be detrimental to oocyte viability in culture,[8,9] Hanks' salts M199 is generally reserved for short-term culture or handling in room atmosphere, while Earle's M199 is more commonly used for longer culture and maturation in a 5% CO_2 atmosphere.

Immature oocytes oocytes will attempt to resume meiosis once removed from the follicle, but require additional hormones for most effective maturation. Formulations for in vitro maturation (IVM) media vary but invariably include follicle stimulating hormone (FSH). Conversely maturation of IMM oocytes can be delayed by holding them at room temperature overnight for one to two nights. This can be particularly useful for appropriate timing of ICSI in the laboratory, since most IVM protocols call for 30 hours of maturation, helping to limit laboratory procedures to normal business hours. The ability to delay maturation also greatly facilitates transport of these oocytes to an ICSI laboratory. Holding at room temperature without the benefit of a CO_2 atmosphere is usually accomplished with either EH medium[10] (a combination of 40% Earle's salts M199, 40% Hanks' salts M199, and 20% serum) or embryo holding medium.[11]

Temperature maintenance is critical for the preservation of the meiotic spindle in oocytes following GV breakdown. While some repair may be possible over time, a decrease in temperature for 1.5 minutes to 32°C can depolymerize the meiotic spindle.[12-14] Reduced culture temperature in a DSF oocyte shipping protocol has been shown to dramatically reduce ICSI blastocyst production, even when oocytes were allowed time for spindle repair.[11]

What techniques are applicable to clinical practice?

Oocyte transfer (OT) is the procedure most applicable to clinical practice if the entire procedure is to be carried out in an individual practice. Oocyte transfer requires the ability to recover DSF oocytes, maintain them in appropriate conditions prior to transfer, the availability of a synchronized recipient whose own oocyte has been removed or a hormone-treated non-cyclic recipient mare, good quality semen, and the ability to perform surgical transfer of the oocyte into the recipient oviduct. With practice, many practitioners can successfully perform this procedure. It is however, logistically demanding, time consuming, and can be recipient mare intensive. Like all ART procedures results can be significantly affected by mare age and oocyte quality.[6,15]

Oocyte recovery and transport to a laboratory for ICSI is probably applicable to many more practice situations. This allows the veterinarian to recover oocytes from client mares with reproductive abnormalities as well as for the production of embryos from semen of limited availability or in vivo fertility. Practitioners equipped for embryo transfer need only add a few pieces of equipment in addition to practice time to recover useful oocytes. Recovery of IMM oocytes can be scheduled in advance on a regular basis, simplifying addition to a busy practice schedule.[16] Protocols for transport of both DSF and IMM oocytes for ICSI have been shown to provide similar results to on site collection and ICSI, and are currently being successfully commercially used.[5,11]

The establishment of ICSI program itself can be a quite daunting project for any practice and should not be entered into lightly. The equipment expense and technical skills are significant, but even without these considerations, the creation of a complete successful laboratory program is an elusive goal that has escaped many. The ability to create and maintain an effective ICSI program that is a service to clients is probably not practically attainable in most situations.

Oocyte recovery

Oocyte recovery is a limiting factor in any ART program, the methods will vary according to practitioner preference and the type of oocyte required. Flank aspiration of DSF oocytes for either OT or ICSI using a trocar, cannula, and needle has been shown to be highly effective, and requires very little equipment.[17] It is of limited usefulness however for aspiration of IMM oocytes from small follicles. Collection of oocytes immediately following the death or euthanasia of mares by scraping follicles with a

bone curette can provide a useful service to mare owners, since the GV oocytes recovered can be held and transported at room temperature in a less harsh environment than in a disembodied ovary. The most versatile technique for oocyte recovery is ultrasound guided transvaginal follicular aspiration (TVA) since this technique allows for collection of oocytes from both dominant and small follicles under ultrasound visualization. It does however require additional equipment and some time to develop the skills for proficient oocyte collection. The complete TVA procedure has been described.[18]

Transvaginal aspiration TVA utilizes an ultrasound probe holder and needle guide to allow the operator to place the ultrasound probe and ovary in close proximity, separated only by the vaginal wall. This provides the visualization necessary to accurately direct a needle into follicles 6mm and larger. Practitioner preference and equipment availability will influence the selection of micro-convex or linear ultrasound probe. The orientation of the micro-convex probe allows easier access to ovaries of mares with short suspensory ligaments and vaginal vaults while the linear probe may allow the operator additional surface area to stabilize the ovary for needle penetration. A double lumen 12 gauge needle is used to allow simultaneous aspiration and flushing of the follicle. Suction is maintained with a vacuum pump regulated to -150mm mercury.

Transvaginal aspiration will vary somewhat depending on the follicle being aspirated. Since DSF oocytes are very temperature sensitive, warmed flushing solution (usually heparinized embryo flush medium) and warmed collection bottle, search dishes, and microscope stage are warranted. Since DSF are generally aspirated 24-30 hours following gonadotropin stimulation, the attachment of the DSF COC to the follicle wall is decreased relative to those in IMM follicles. Turbulence caused by infusion of flushing medium and by manual manipulation of the follicle and aspiration needle facilitates dislodging and collection of the COC. Conversely, aspiration of IMM follicles requires turbulence and scraping of the follicle wall with the aspiration needle for good recovery, as the COC is more compact.

Transvaginal aspiration with successful and efficient oocyte recovery takes not only an experienced operator and proper equipment, but also assistants that are well versed in the procedure. The operator may handle not only the ultrasound probe and the rectal manipulation of the ovary, but the needle advancement and placement itself. Other practitioners prefer to have a separate individual handling the needle. While a foot pedal may operate the aspiration pump, the infusion of flushing medium requires another person with the ability to read the ultrasound image to provide adequate filling and refilling of each follicle.

Oocyte transport

Oocyte transport can provide clients access to ART programs through their reproductive veterinarians. Mares that otherwise would be retired or shipped to an ICSI facility can stay in the care of their veterinarian and have oocytes recovered and shipped to an ICSI facility. One study showed no reduction in blastocyst production rates utilizing transport protocols compared to the standard on site protocols.[11] Timing as well as temperature and pH management are critical in the collection and transport of the DSF oocyte since meiosis has resumed following gonadotropin stimulation. The timing of stimulation will dictate the timing of collection and transport so that the oocyte arrives at the ICSI laboratory prior by 40-42 hours following stimulation at a time when the laboratory is prepared to perform ICSI. Oocytes from DSF have the highest rates of blastocyst formation, but the timing and transport requirements can be difficult to fit into some practice environments. Collection, shipping temperature and timing requirements of IMM oocytes are somewhat more forgiving, while aspiration of small follicles may be somewhat more difficult for the beginning aspirator.

What to expect

Initiation of an oocyte recovery program for ART can be both a stimulating and frustrating proposition for the practitioner. The ability to deliver a greater level of service to the client by utilizing new techniques can be quite rewarding. Conversely the learning curve and time required to develop these techniques is somewhat surprising to many individuals. These are the first things to expect.

Transvaginal aspiration oocyte recovery rates for an experienced operator should be around 70% or more per DSF follicle.[6,15] This is dependent on appropriate timing of gonadotropin injection, the response by the mare, and aspiration skill. Oocyte recovery from small follicles should be generally around 60% overall,[16] but this is influenced dramatically by follicle size, with the highest recovery rates from follicles < 20mm. The number of IMM oocytes recovered is dependent on both the number and size of the small follicle cohort present on the ovaries of an individual mare. This in turn is influenced by the season of year, the breed, the age, and prior aspirations of the mare. Older mares will tend to have a smaller follicular reserve and subsequently fewer follicles present on the ovaries. Follicles tend to be more numerous during transition, although this is variable between individuals.

Blastocyst production from oocytes submitted to ICSI will vary from mare to mare and also between stallions. Research situations can produce blastocysts from 70% of DSF oocytes and 35% of IMM oocytes,[11] but clinical results are lower than this in most situations, relative to mare age, oocyte quality, and semen quality. Based on this author's experience, overall production of 35% from DSF oocytes and 20% from IMM oocytes may be anticipated with wide variations for individual stallions and mares.

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The equine embryo
Advances in techniques: trophectoderm biopsy and embryo vitrification

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Introduction

The commercial availability of equine embryo transfer has recently led to tremendous growth in breeder's requests for desired genotypic and phenotypic specificity of offspring as well as embryo cryopreservation. These scientific advances come at a most opportune time for the horse industry because producing desired offspring not only reduces the "unwanted horse" population, but it favors a decrease of inherited disease, as well as permits the preservation of desired embryos until an owner can provide an optimal environment for their development.

Trophectoderm biopsy and preimplantation genetic diagnosis

Accurate gender identification of the equine fetus can be readily accomplished by transrectal ultrasonographic examination after 60 days of gestation. If the gender is not acceptable to an owner, the mare is often given an abortifacient agent and the unwanted fetus is aborted. While some consider abortion at this stage of gestation based on gender to be unethical, it should also be recognized that the practice is reproductively inefficient as well. Not only are pregnant recipient mares in use for at least 60 days carrying a fetus of unknown gender, but if aborted, they may fail to cycle again during the breeding season due to endometrial cup formation and the continued secretion of equine chorionic gonadotropin (eCG). Furthermore, the semen used to produce these pregnancies is wasted-it may be in limited supply, whereby the breeding dose for one mare precludes the use in another, or very valuable, as is often the case with frozen semen. Furthermore, stallion owners may not be aware of or in agreement with the abortion of their stallion's offspring due to gender preference.

Although sex-sorted semen has the potential to produce the desired gender, its use has not yet become commercially available in most parts of the world, nor does the processed sperm appear to tolerate cryopreservation. Therefore, trophectoderm (TE) biopsy of an embryo followed by preimplantation genetic diagnosis (PGD) represents an excellent alternative for early gender selection and heritable disease surveillance. The TE biopsy procedure requires specialized equipment that is capable of micromanipulation of the embryo as well as experienced technicians, for puncture and biopsy of the expanded blastocyst can be challenging.

The unique equine capsule, formed by *in vivo,* but not *in vitro*-produced embryos, poses a significant barrier to obtaining trophectoderm cells and must be effectively penetrated so as not to compromise embryo viability during the biopsy procedure. The laser-assisted biopsy procedure used in other mammalian embryos (Figures 1, 2), humans included, has been largely ineffective in penetrating the equine capsule and maintaining viability of the embryo. The earliest report of an equine embryo biopsy and successful pregnancy following transfer was published by Huhtinen et al.[1] These researchers used a microblade to obtain cells from Day 6 embryos. The pregnancy rates following transfer were low in this study, with only 3 out of 16 biopsied embryos resulting in a pregnancy. Several more recent studies have followed, reporting an improvement over these initial pregnancy rates following embryo biopsy. Seidel et al[2] compared pregnancy rates following the microblade embryo biopsy technique versus aspiration of TE cells using a glass 6-10um glass pipette. They reported a pregnancy rate of 40% following the microblade biopsy technique versus 28% for embryos >300um diameter and 75% for embryos < 300 um diameter following aspiration. These authors concluded that both the biopsy technique and the size of the embryo contributed to pregnancy rate, and that embryos <300um diameter had a more favorable outcome following needle aspiration. However, Choi et al[3] reported normal pregnancy rates following biopsy and transfer using a piezo drill and aspiration needle biopsy technique in embryos that ranged in size from the morula stage to the expanded blastocyst stage (up to 1300um in diameter). Herrera et al[4] recently reported similar results; they found no significant difference in 25 day pregnancy rates between transferred biopsied embryos versus nonbiopsied embryos (59% versus 62% respectively), nor any

difference based on embryo size in either group. Furthermore, in this latter study, the capsule was breached without the use of a piezo drill.

The piezo drill is an expensive piece of equipment and is not necessary for successful equine trophectoderm biopsy and resultant pregnancy, even when used with *in vivo* produced large blastocysts. Using a micromanipulator with a large holding pipette to secure the embryo, a 25 micron inner diameter glass micropipette or polar body biopsy needle,[a] one can penetrate the capsule and retrieve sufficient numbers of cells from the trophectoderm by scraping and aspiration (Figure 3).

From a commercial viewpoint, it is important to note that embryos can be held overnight in warmed holding medium prior to biopsy[3] as well as held for as much as 7-10 hours after biopsy without affecting viability.[4] Therefore, embryos can be recovered at one farm, shipped overnight for biopsy at a laboratory, and then returned or shipped elsewhere for transfer into a surrogate mare. Herrera et al[4] reported that PCR gene amplification of biopsied cells required 6 hours for gender determination, so ideally only embryos of desired gender would be transferred. In this study, however, the researchers were only able to determine gender in 50% of their samples (52 of 104 samples) and the specificity of their assay for females was 84.3%.

Presently, the Veterinary Genetic Laboratory (VGL) at the University of California-Davis (https://www.vgl.ucdavis.edu/services/horse.php) provides a service to detect not only gender, but also several lethal or crippling inherited diseases, from biopsied TE cells with >97% accuracy. Through a process of whole gene amplification, specific gene loci for HERDA, HYPP, lethal white, cerebellar abiotrophy, etc are reliably identified. Furthermore, prediction of coat color of the resultant offspring is also possible. Unfortunately, at the time of printing, the detection of combined immunodeficiency (CID) loci is under patent protection and the holders will not grant a license to UC Davis to test embryos for this disease.

Embryo vitrification

An unforeseen, but ultimately beneficial, consequence of trophectoderm biopsy is blastocoel fluid aspiration and rapid collapse of the blastocyst (Figure 4). This discovery has revolutionized equine embryo vitrification techniques and the successful cryopreservation of expanded equine blastocysts.

Vitrification provides an attractive option for many in the breeding industry under the following circumstances:

1. A synchronized recipient mare is unavailable.
2. To hold an embryo while awaiting PGD results.
3. Allows multiple embryos to be collected (via embryo flush or oocyte collection) without the expense of a synchronized recipient mare.
4. Sale of the vitrified embryo.
5. International distribution of genetics.
6. Enhance genetic variability and hybrid vigor.
7. Assess offspring phenotype-"temporary genetic banking"
8. Late or offseason cycling mares can produce embryos for more desirable transfer date
9. Unexpected double or triple ovulation
10. Preserve genetics (in euthanized mares).
11. Production of embryonic stem cells

Until several years ago, successful pregnancy following vitrification was limited to embryos that were <300 um in diameter. These embryos were most often recovered from donor mares 6-6.5 days following ovulation and were at the late morula or early blastocyst stage of development. This size limitation affects reproductive efficiency for two reasons. First, embryos at this stage, even before vitrification, have been shown to have a lower pregnancy rate and higher rate of early embryonic loss when compared to later stage blastocysts.[5,6] Furthermore, the recovery rate of day 6 embryos has been shown to be significantly lower than that of day 8 embryos,[6] presumably because these early embryos

have not yet entered the uterus. Therefore, delaying an embryo flush until day 7 or day 8 offers an improved rate of embryo recovery success.

An early study[7,8] reported 16-day pregnancy rates of 67% after day 6 embryos were vitrified in 0.25 ml straws, warmed and then transferred into the uterus of recipient mares. These researchers exposed embryos to increasing concentrations of ethylene glycol and glycerol (EG/Gly) vitrification solutions before loading the embryos into straws. Based on this study, vitrification kits and a standard vitrification technique became available commercially. However, using commercial protocols, several studies did not report pregnancy rates as high as reported in the initial study. Troedsson et al[9] reported that only a single pre-capsule embryo (1/8) that had undergone biopsy and vitrification survived to day 45 pregnancy in a recipient mare. Choi et al[10] reported that, using the standard EG/Gly protocol for vitrification following biopsy, no day 6 embryos (0/5) resulted in pregnancy at 16 days.

In this latter study, the researchers also explored alternative methods for vitrification for embryos of different sizes and developmental stage. Their work with successful TE biopsy in expanded blastocysts had resulted in the serendipitous discovery of blastocoel collapse. Scherzer et al[11] attempted to vitrify embryos by replacing blastocoel fluid with cryopreservation solution using a laser system to penetrate embryonic capsule; however, only 1/9 recipient mares was pregnant at 23 days after transfer . Campos-Chilton[12] et al also studied an alternative method using ethylene glycol for vitrifying and warming very young equine embryos (two-eight cell stage) before transferring them into the oviduct of recipient mares. This group vitrified the embryos in open pulled straws, which limited the final volume to 1-2ul of vitrification media that bathed the embryo. The pregnancy rate was 62% (6/8) at day 20 following transfer. Using the same protocol and medium for vitrification, a sucrose gradient described by Sun et al[13] for warming, and a minute volume of media to surround the embryo, Choi et al[10] reported an 86% pregnancy rate (6/7) from expanded blastocysts 407 to 565 um in diameter that were collapsed, vitrified and warmed before transfer. Instead of loading the embryos into an open pulled straw, these researchers loaded the collapsed embryo into a fine-diameter microloader tip[b] as described by Sun et al[13] before plunging the pipette into liquid nitrogen. When warming the embryo, the tip of the microloader was immersed in a warmed drop of thawing medium, releasing the embryo.

This author has found similar pregnancy rates using the protocol described by Choi et al for the vitrification and warming of collapsed blastocysts as well as earlier stage embryos. However, the equipment is different. The embryos are loaded onto a beveled trough of a device called the Cryolock®[c]. The trough of the Cryolock® tip holds the embryo in place, surrounded by less than 0.2 ul final vitrification medium at the time of plunging into liquid nitrogen. When warming, the tip of the trough is immersed into a 10 ul warmed drop of the initial sucrose warming solution and agitated gently, releasing the embryo. The author has found that a healthy embryo gradually reexpands over the next 30 minutes during transport in a warmed Equitainer®[d].

Summary

The research cited in this paper has transformed the equine embryo transfer industry, especially with respect to preservation of valuable and healthy equine genetic lines. The ability to successfully biopsy and cryopreserve early equine embryos up to the expanded blastocyst stage of development allows not only selection of offspring by breeders but enhances their commercial enterprise and avails for a healthier population of horses.

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[b]Eppendorf NA Happauge NY
[c]Biotech Inc, Alpharetta, GA
[d]Hamilton Research Inc Ipswitch, MA

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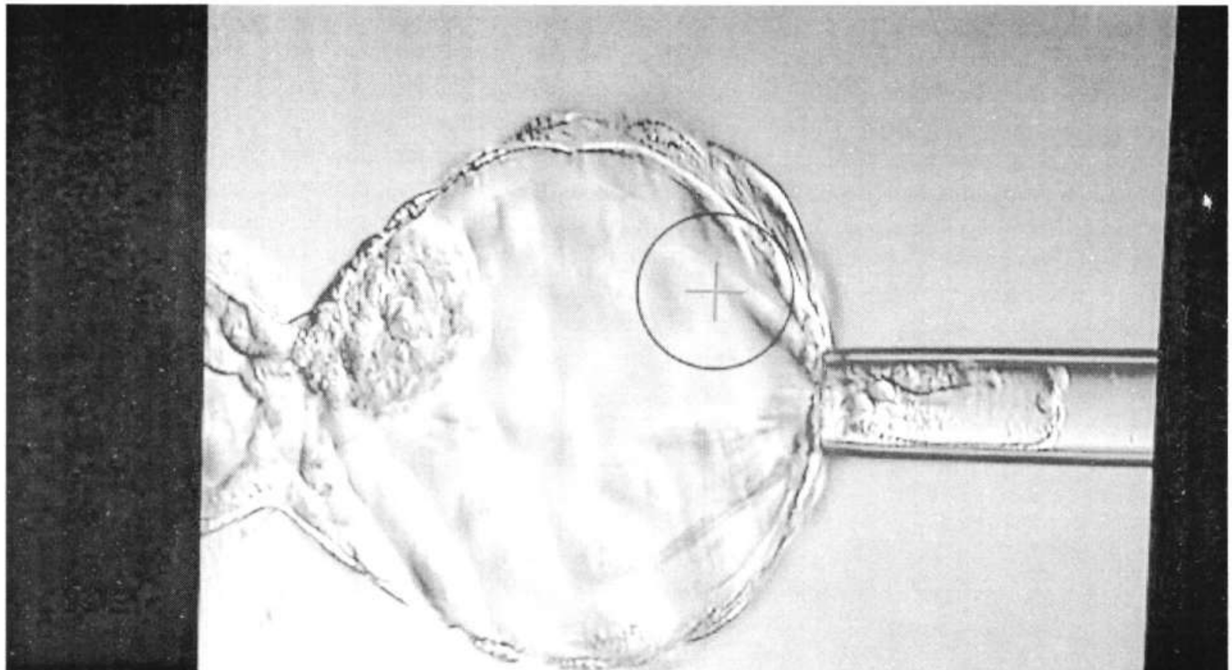

Figure 1: Mouse trophectoderm biopsy following laser assisted penetration. Courtesy of Keith Masterson, MS.

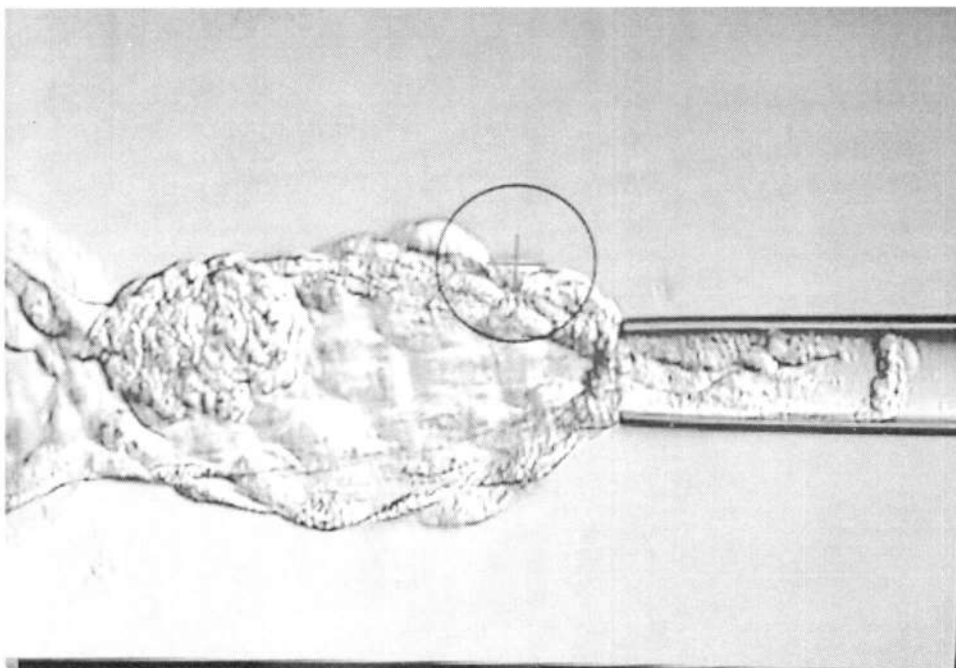

Figure 2: Mouse trophectoderm biopsy. Cells are gently aspirated into the biopsy pipette. Courtesy of Keith Masterson, MS.

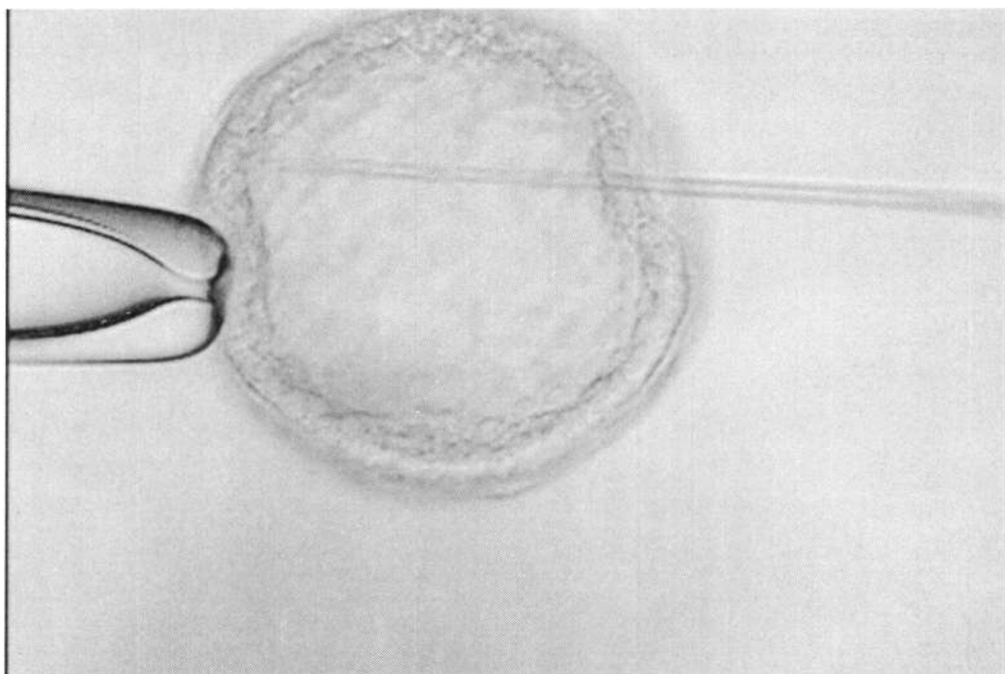

Figure 3: Trophectoderm biopsy of an equine day 8 expanded blastocyst.

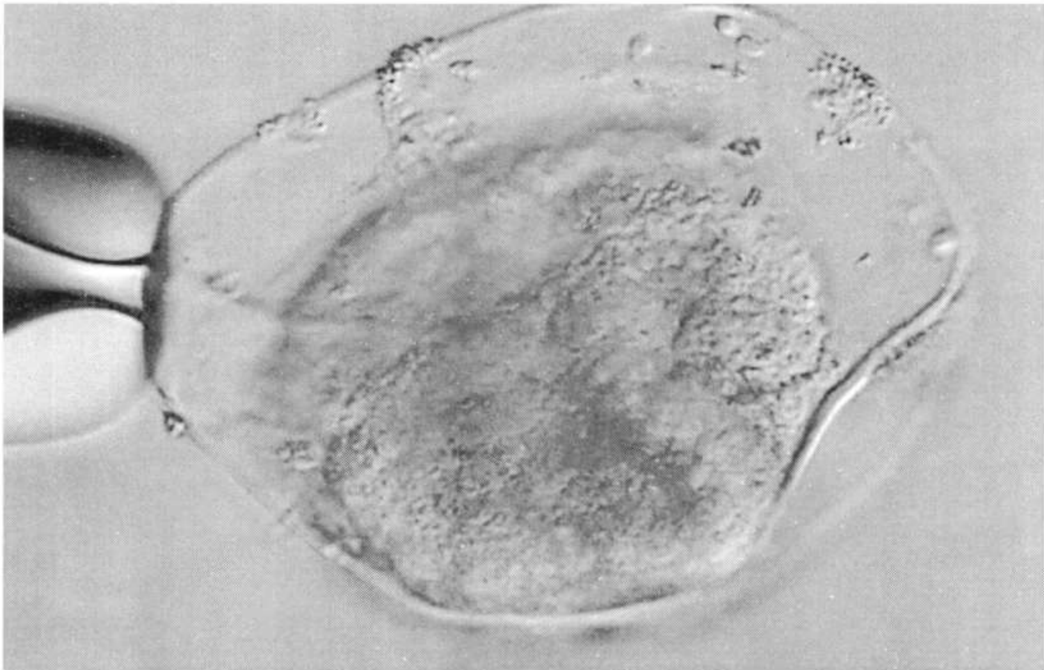

Figure 4: A collapsed day 8 expanded equine blastocyst following blastocoel aspiration, preparing for vitrification.

(Editor's note: Photographs in this manuscript are available in color in the online version of Clinical Theriogenology.)

Immunology and immunomodulation of the reproductive tract

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Introduction

Persistent endometritis is a major cause of reproductive wastage and hence economic loss to the equine breeding industry. The endometrium of the mare is periodically exposed to numerous irritants and potentially infectious agents during breeding, iatrogenically during veterinary procedures, and when normal defense mechanisms become incompetent.

Multiple physical and functional barriers exist to prevent or minimize contamination between episodes of breeding and during pregnancy. Once introduced to the endometrium infectious agents encounter both innate and adaptive immune defenses consisting of both cellular and humoral factors. Components include the epithelial mucus barrier, resident innate immune cells, and their pro-inflammatory secretions that minimize the risk of infection. Acting in concert with the innate immune system, the adaptive immune system includes both cell-mediated and humoral responses generated towards specific pathogens. Cells of the innate and adaptive immune systems are influenced by the ovarian sex hormones, local growth factors, and the commensal genital tract flora. Much of what is known has been determined from laboratory species, humans and production animals. In the current review, this will be extrapolated to the horse where applicable.

Background

The reproductive tract of the mare is a dynamic organ system, rhythmically changing under the influence of estrogen and progesterone of ovarian origin. While providing protection against sexually transmitted pathogens and environmental contaminants, it must tolerate commensal flora present in the lower reproductive tract. The endometrium must also allow for tolerance of sperm, develop an appropriate and limited inflammatory response following mating to initiate uterine clearance and increase chances for conception, provide an environment for fertilization and implantation of the conceptus, and facilitate growth of the genetically distinct fetus.

The tubular female reproductive tract can be divided into a number of regions with differing structure and function. These include the oviducts, uterus, cervix, vagina and vestibule. The mucosal lining varies between these regions, but all consist of an epithelial lining with underlying stroma which display a cycle-dependent structure composed of mucous glands, vascular tissue, and cellular components. This mucosal barrier to infection is not merely physical, but instead consists of innate cellular and chemical responses to the invasion of potential pathogens. These responses vary with the region of the tract involved.[1] During natural mating, stallions ejaculate into the uterus, thus placing the burden of immunological response post-breeding on the endometrium.

Resistance of the mare endometrium to the establishment of persistent endometritis has historically been considered the result of physical clearance of irritants and an appropriate local inflammatory response. Physical clearance requires an appropriate disposition of the genital tract [2], competence and function of anatomical barriers,[3,4] myometrial contractility,[5-7] lymphatic drainage,[8] and mucociliary clearance.[9,10] Local cellular and humoral aspects of the immune system have been widely investigated. These include immunoglobulin and complement concentrations,[11] and polymorphonuclear neutrophil function.[6,12,13]

Treatment of persistent endometritis has sought to overcome deficiencies in the immune response of the uterus as a whole, and has varied over time as more has become known about the factors operating. Restoration of appropriate external genital anatomy,[3] augmentation of uterine clearance,[5] and treatment of specific infections with antimicrobials and/or chelating agents[14] have become the mainstays of current treatment, although for some time deficiencies in function of both the humoral and cellular immune response to endometrial contamination were thought responsible for the persistence of endometritis.[15,16] However, impaired uterine defenses were shown not due to a local immunoglobulin deficiency.[11]

Responses to endometrial challenges

Local innate immune responses

Mucosal epithelial cells form a physical and immunological barrier, initiate signaling of the underlying immune system, are involved in production of cytokines and chemokines, participate in apoptosis and phagocytosis of infected cells, enable activation of the adaptive immune response, and initiate an acute inflammatory reaction in response to irritation or infection.[17] The mucosal epithelial barrier is affected by cytokines, ovarian hormones, Toll-like receptor (TLR) agonists, and the presence of pathogens.[18] In addition to the epithelial cells themselves, the mucosal barrier in augmented by a mucus layer preventing direct contact with potential pathogens [19]. Mucus consists of water and mucin, a high-molecular-weight glycoprotein that can trap microorganisms.[20] Amount of mucus and its properties vary depending on sex hormones. Estrogen dominance leads to mucus that is less viscous and more favorable for sperm migration, whereas mucus during progesterone dominance is comparatively thick, sticky, and hostile to the passage of sperm into the uterus.[21]

During the initial stages of the immune response, antigen presenting cells (APC) which include dendritic cells and macrophages express a multitude of surface molecules which non-specifically recognize pathogen-derived molecules or pathogen-associated molecular patterns (PAMPs). The best known of these surface molecules are a family of transmembrane proteins known as the Toll-like receptors (TLRs), however a number of other surface molecules performing a similar function have been elucidated.[22] Exogenous molecules recognized include bacterial lipopolysaccharides (LPS), lipoproteins and peptidoglycans, and viral RNA and DNA. The TLRs are able to respond to a diverse array of structures, however they may require simultaneous binding to other endogenous cell surface receptors (e.g. in the case of TLR4 binding to CD14 is necessary to bind LPS) to respond. Once engaged, the TLRs initiate downstream signaling by recruitment of one or more adaptor proteins, the MyD88-dependent cascade which leads to secretion of pro-inflammatory cytokines, or the TRIF-dependent cascade which results in production of type 1 interferon (IFN) in addition to inflammatory cytokines and chemokines. These in turn promote genomic protein transcription by NF-κB and initiation of a non-specific immune response.[1] TLR4 is involved in the recognition of lipopolysaccharide on the surface of *Escherichia coli*, commonly isolated from mare endometritis cases.[23] Response to bacterial pathogen invasion may be different however as TLR4 expression by the endometrium in one equine study was not altered by insemination with live sperm.[24]

Toll-like receptors and antimicrobial peptides have been demonstrated in the bovine endometrium.[25] The response of the endometrium to bacteria and their purified PAMPs has been investigated using *ex vivo* bovine endometrial explants, with IL-6, IL-1β and IL-8 produced with the inflammatory response dependent on the stage of the estrous cycle.[26] In the equine, endometrial mRNA expression of pro-inflammatory cytokines (IL1-β, IL-6, TNF-α and IL-8) increases and anti-inflammatory IL-10 decreases after insemination with killed sperm.[27,28] Neutrophil chemotaxis requires IL-8 which is expressed in higher levels in mares susceptible to endometritis compared to those resistant during estrus.[28]

Complement cleavage factor C3b has been demonstrated in mare uterine secretions during infection.[16,29,30] In one study complement was shown to be an important contributor to opsonization activity in uterine secretions preceding an inflammatory response.[31] The complement system may directly lyse the infectious agent. Other inflammatory products include $PGF_{2\alpha}$, secreted in response to uterine inflammation and correlated to neutrophil numbers.[32,33] Acute phase proteins are also present, with α1-antitrypsin recovered from uterine flushings however this was not increased following induction of bacterial endometritis, and was instead related to cycle stage with levels in estrus greater than diestrus.[34] As levels were correlated to albumin present in uterine fluid, a systemic origin of α1-antitrypsin was determined.

Antimicrobial peptides have also been demonstrated in the reproductive tract, and are considered an ancient mechanism of the innate response.[35] In addition to microbiocidal activity they affect cytokine induction, chemotaxis, cell proliferation, and modulate both innate and acquired immunity.[36] These

compounds act synergistically and have redundancy of function enhancing protection over any single factor.[37] Members include lactoferrin, the defensins, elafin, cathelicidin, and lysozyme, being produced by both epithelial cells and neutrophils[35] with regulation by bacterial activity, hormonal status and inflammation.[38]

Epithelial cells, macrophages, natural killer cells, and neutrophils produce cytokines and chemokines. Chemokines are cytokines with potent local chemotactic properties, recruiting leukocytes to site of inflammation.[39] Subsequent to this, local cytokines enable the differentiation and activation of the attracted leukocytes.[40] Secretion has been shown experimentally in other species to be preferentially to the cell apex or uterine lumen creating a gradient along which to attract cells to the endometrium.[41] Additionally, bactericidal and virucidal agents are enhanced, these providing a system of defense during times the adaptive immune response is downregulated, particularly by sex hormones during pregnancy.

Innate cellular responses are provided by dendritic cells (DC), macrophages, and natural killer (NK) cells. Dendritic cells are essential for induction of the immune response, prevent infection by direct inactivation, and as the major APC of the reproductive tract provide a link between innate and adaptive immunity. Location is variable, being the subepithelial stroma of the endometrium and epithelium of the vagina.[42] Exposure to pathogens and inflammatory stimuli, such as lipopolysaccharide (LPS), leads to maturation of DC which can then enable the development of T-helper 1 (Th1) cells.[42] Function and differentiation of DC is the result of local cytokines, chemokines and ovarian steroids.[43] In addition to induction of a protective immune response, DC are also integral in the induction of immunological tolerance essential for the maintenance of pregnancy.[44] Macrophages are involved in inflammatory responses, removal of debris, pathogen recognition, and stimulus of the immune system via production of cytokines and chemokines. Widely distributed throughout the reproductive tract, migration into the endometrium is influenced by estradiol and progesterone.[45-47] Macrophage proliferation and function is influenced by estrogen,[48] while phenotype reflects the local environment.[40] Natural killer cells are key innate immune cells, both promoting the immune response and by secretion of cytotoxic compounds eliminating virus-infected and neoplastic cells.[49] The inflammatory response is amplified, macrophage activation promoted, cytotoxic T cells are generated, and cytokines produced.[50] Within the uterus, specialized uterine NK cells (uNKs) express multiple TLR and produce cytokines in response to TLR agonists further activating of innate immunity.[49,50] Eosinophils are occasionally seen within the endometrium and have been associated with failure of anatomical barriers and thus pneumometra.[51] Eosinophils have also been associated with fungal infection.[52]

Local adaptive immune responses

The adaptive immune response is initiated by specific pathogens. Following pathogen capture and processing of antigens (innate immune response) APCs present the non-self ligands in conjunction with MHC class 1 and 2 molecules. The APC themselves are highly secretory promoting both local and systemic inflammatory and immune responses. With the benefit of expression of costimulatory molecules also induced by the non-self antigens, APCs are involved in the activation of both specific B and T lymphocyte function (adaptive immune response). Lymphocytes in turn crosstalk with activated macrophages to promote efficient phagocytosis. Adaptive immunity includes Th_1 (cell-mediated), Th_2 (humoral), and T regulatory responses. Th_1 based immunity is driven primarily by T lymphocytes and results in destruction of intracellular pathogens. The CD8+ cytotoxic T cells target infected cells via MHC class I molecules on the cell surface, inducing apoptosis and cytolysis.[53] High levels of IFN-γ are secreted by CD4+ T cells which regulates cytotoxic T cells while also blocking viral replication.[54]

Humoral immunity is based on the production of specific antibodies that bind antigens (cell-associated or free) interfering with cellular invasion or directly neutralizing the pathogen. Phagocytosis by macrophages is enhanced. The transformation of B cells to plasma cells is facilitated by the CD4+ helper T cells.[55] Antibody production in the genital tract is influenced by ovarian activity. In the uterus, IgA and IgG are experimentally elevated in rats by estradiol.[56] In contrast, vaginal antibody levels are decreased.[57,58] In response to estrogen, IgG moves down a concentration gradient from blood to tissue

before entering the luminal fluid, either by paracellular diffusion or receptor facilitated means. In contrast, IgA travels against a concentration gradient as in the male.[59] Difference in class, anatomical distribution, and antibody level implies compartmentalization in the immune function throughout the female reproductive tract, with modifying hormonal influences. Furthermore, these responses must be tailored to provide specific pathogen protection while allowing maintenance of the fetal allograft.

Antibodies, IgA and protein levels were investigated in mares categorized as infected by bacteriology and supportive cytological findings.[60] Infected mares were found more likely to have elevated levels of IgA and protein, while levels of IgG were not increased. Progesterone treated acyclic mares were found to have higher numbers of bacteria and IgA compared to control mares, further supporting the finding of increased IgA in infected mares.[7]

Systemic inflammatory and immune responses

Serum amyloid A (SAA) concentration has been widely investigated as a systemic marker of inflammation in the horse.[61-63] Uterine inflammation is a normal post-breeding event in the mare.[4] Histologically normal endometrium of the mare has been shown to constitutively express mRNA for SAA in moderate levels.[64] Conflicting information exists regarding a detectable systemic SAA response to endometritis as in one study a systemic increase in levels was not found [24] however in another plasma SAA levels were found significantly correlated with endometrial SAA gene expression.[65] In experimentally induced placentitis, SAA was found to be elevated within 96 hours after intra-cervical inoculation of *Streptococcus zooepidemicus*.[66]

Response to breeding

Spermatozoa in the uterus induce rapid chemotaxis of polymorphonuclear cells that are detectable in the uterus as soon as 0.5 hours after AI and peak at 4-8 hours after AI.[67] Endometrial mRNA expression of pro-inflammatory cytokines (IL1-β, IL-6, TNF-α and IL-8) increases and anti-inflammatory IL-10 decreases after insemination with killed sperm.[27,28] In a study comparing the inflammatory response to frozen semen and extender alone, the luminal neutrophil response was found to be greater in mares inseminated with the frozen semen, while macrophages, lymphocytes and plasma cells did not differ between the groups.[24] Concentrations of prostaglandin (PG) F2α increased 16 hours after both frozen semen and extender treatments, SAA did not change with treatment, and IL-8 and TLR4 (the key receptor of pathogen associated molecular patterns) had no changes in expression.[24]

Mares susceptible to post-breeding inflammation have higher basal levels of mRNA expression of proinflammatory cytokines compared to normal mares.[27] At 24 hours after insemination, susceptible mares also have increased mRNA expression of IL8, a neutrophil chemoattractant, and lower expression of IL10, an inflammation modulating cytokine.[28,68]

In another study evaluating endometrial inflammatory markers within the first 24 hours after breeding, differences were detected between susceptible and resistant mares 6 hours after insemination. Resistant mares had higher mRNA expression of IL-6, IL-1 receptor antagonist and IL-10 compared to susceptible mares, while susceptible mares had an increased numbers of polymorphonuclear cells 2 and 12 hours after insemination when compared to resistant mares.[69] This suggests a deficiency in the immunomodulatory response in mares susceptible to post breeding endometritis.

Additional studies have evaluated uterine nitric oxide (NO) production associated with breeding induced endometritis [70]. During inflammation, nitric oxide is produced by inducible nitric oxide synthase (iNOS), a calcium independent mechanism of producing large amounts of nitric oxide. Inflammatory signals such as IL-1 and IFN-γ lead to transcription of iNOS. Effects of NO include removal of pathogens and smooth muscle relaxation. These studies revealed differences in intrauterine NO between mares that are sensitive to or resistant to breeding induced endometritis however it remains unclear whether this difference is a cause or an effect of endometritis.

Treatments to modulate the immune response in the non-pregnant mare

Glucocorticoids

Glucocorticoid use has been reported as a successful method of controlling post-breeding endometritis. Compounds investigated include prednisolone acetate (0.1 mg/kg)[71] and dexamethasone (50 mg IV per horse).[72] In a fertility trial evaluating the use of prednisolone, thirty cycles of fifteen mares were evaluated comparing frozen semen insemination without immunomodulatory treatment followed by insemination with corticosteroid therapy. Corticosteroid therapy consisted of 0.1 mg/kg prednisolone acetate administered when a 35 mm follicle and endometrial edema were present, followed by repeat administration every 12 hours until ovulation was detected. In this trial, none of the mares achieved pregnancy without prednisolone treatment and 64.5% achieved pregnancy with prednisolone treatment.[71]

Dexamethasone (0.1 mg/kg IV) administered once at the time of breeding was evaluated for efficacy in reducing breeding induced inflammation and increasing pregnancy rates in susceptible mares. Dexamethasone decreased post-breeding small volume lavage efflux turbidity and endometrial edema. Dexamethasone did not alter the pregnancy per cycle rate, however in mares that were determined to be at high risk of developing post-breeding endometritis with at least three of the study's risk factors, pregnancy rates were significantly improved. Risk factors included: abnormal reproductive history, positive endometrial culture, at least 2 cm endometrial fluid prior to breeding, abnormal perineal conformation or unrepaired Caslick's surgery after foaling, abnormal cervix, greater than 1.5 cm post-breeding fluid, post-breeding fluid persisting beyond 36h, and abnormalities of the reproductive tract.[72] A recent study evaluated use of 0.1 mg/kg IV dexamethasone with intrauterine infusion of 10^5 CFU of *E. coli* to assess changes in pro- and anti-inflammatory cytokine expression.[73] Treatment with dexamethasone caused a significant effect on endometrial expression of cytokines and SAA in susceptible mares. Of the pro-inflammatory cytokines, decreased expression of IL-1β was observed, an increased expression of IL-6 was noted immediately after dexamethasone administration, and IL-8 (a potent chemoattractant for transepithelial migration of PMNs into tissue) expression was decreased. The anti-inflammatory cytokines IL-10 and IL-1ra had increased expression following dexamethasone administration.[73]

Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in clinical practice to manage inflammation associated with breeding, embryo transfer, and placentitis. In a study evaluating the COX-2 inhibitor vedaprofen, barren mares (n=8) with a history of post-breeding endometritis were treated (2 mg/kg PO initial dose, 1 mg/kg PO BID) beginning the day before artificial insemination and continuing until 1 day after ovulation. These mares were compared to an untreated group though all mares received oxytocin. This study found a significantly improved pregnancy rate among mares treated with the COX-2 inhibitor around the time of breeding.[74]

Immunostimulants

Mycobacterium phlei cell wall extract (MCWE) is available as Settle (Bioniche Animal Health, Athens, GA) with the licensed indication as an aid in the treatment of equine endometritis caused by *Streptococcus zooepidemicus* by enhancing the innate humoral immune response. In a study assessing the endometrial mRNA expression of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in mares resistant and susceptible to post-breeding endometritis, intravenous MCWE treatment at the time of artificial insemination modulated mRNA expression of cytokines such that no differences between the two groups was found.[27] A recent study evaluated use of 1.5 mg Settle with intrauterine infusion of 10^5 CFU of *E. coli* to assess changes in pro- and anti-inflammatory cytokine expression. This study did not find MCWE to significantly modulate the endometrial inflammatory response in contrast to previous studies.[73]

Immunostimulants containing *Propionibacterium acnes* (EqStim, Neogen Corp, Lexington, KY) has been shown to induce a non-specific cell-mediated response predominantly by macrophage activation

and cytokine release. In a study evaluating its efficacy in a clinical setting, 95 mares with cytologic evidence of endometritis were randomely administered *P. acnes* or placebo additional to other veterinary treatments. This study found improved pregnancy and live foal rates.[75]

Plasma

Autologous intrauterine plasma infusion reportedly provides serum-derived opsonins to enhance phagocytic function. One study describes the intrauterine infusion of 100 mL frozen-thawed autologous plasma either as part of active endometritis therapy in conjunction with uterine lavage (n=24) or as a post-breeding infusion in mares with subclinicial endometritis (n=3). Pregnancies were achieved in 62.5% of the first group and 100% of the second group.[76] In a subsequent study, mares with induced mild to moderate lymphocytic endometritis were treated with 100 mL intrauterine plasma daily for 5 days similar to the above report (n=16) or received no intrauterine treatment (n=10). This study revealed no significant differences between treatment and control groups.[77]

A variation of autologous plasma is infusion of platelet-rich plasma (PRP). The use of PRP is widely practiced in orthopedic cases in attempt to expedite healing with growth factors and inflammatory modulators. Recently, practitioners have described its use in the uterus for treatment of post-breeding endometritis. One study evaluated the efficacy of infusion of 20 mL autologous PRP (containing on average 257,000 platelets per microliter) 4 hours after insemination in 15 mares resistant to post-breeding endometritis and 8 mares susceptible to post-breeding endometritis. Parameters evaluated included nitric oxide concentration in uterine fluid, percent neutrophils in uterine cytology, and uterine fluid quantity 24 hours after insemination. In resistant mares, the only significant difference was in percent neutrophils in cytology. In susceptible mares, a significant difference was seen in percent neutrophils, nitric oxide quanity and uterine fluid leading the group to conclude that PRP reduced the inflammatory response after breeding.[78] Another study described improved pregnancy and decreased post-breeding fluid in mares susceptible to post-breeding endometritis following PRP infusion.[79]

Pregnancy

Pregnancy maintenance requires the maternal immune system to tolerate the antigenically different fetal and placental tissue. Mechanisms to achieve this have been divided into three categories: 1) suppression of paternally inherited alloantigens such as MHC antigens; 2) altered maternal immune response during pregnancy; and 3) local immune modulation at the interface between uterus and placenta.[80]

The majority of the allantochorionic trophoblast cells suppress the expression of major histocompatibility class (MHC) II genes that are involved in the activation of CD4+ helper T lymphocytes. Allantochorionic trophoblasts of the horse also fail to express MHC class I molecules that are targeted by alloantibodies and CD8+ cytotoxic T cells. Equine chorionic girdle and early endometrial cup trophoblast cells do express MHC class I molecules, similar to other species in which the most invasive trophoblast cells express MHC class I molecules for reasons that are not completely understood. These MHC class I antigens induce strong maternal antibody responses in early gestation.[80]

While the mare may mount a systemic humoral immune response to paternal MHC class I antigens, the cell-mediated immune response appears to be suppressed. During pregnancy, mare peripheral blood lymphocytes are less able to develop into cytotoxic T lymphocytes.[81] Though this may be advantageous to spare the fetus, there is potential that this may make the mare more vulnerable to certain types of infection. Progesterone also seems to modulate the immune response in the uterus. In experiments in sheep, progesterone supplementation prolonged survival of allografts in the endometrium compared to controls without progesterone supplementation.[82]

The mare exhibits a local immune response in the form of CD4+ and CD8+ T cells surrounding the endometrial cups. Trophoblast cells produce factors that appear to locally modulate the immune response by limiting T cell function or inducing apoptosis.[83] In the majority of mares, endometrial cups have a lifespan of approximately 60 to 80 days, forming at approximately 40 days gestation and persisting until around 120 days gestation. In a recent study investigating the role the immune system surrounding

the endometrial cups plays in their degradation of the cups, equine chorionic girdle trophoblast tissue was transplanted into severe combined immunodeficient (SCID) mice that would be unable to mount an immune response to the transplanted tissue. In these animals, the trophoblast lifespan was approximately the same duration as expected in the pregnant mare, suggesting that the maternal immune response does not play the largest role in elimination of endometrial cups.[84]

In later gestation, placentitis is of significant concern as a cause of fetal loss. In an experimental placentitis model, infection was associated with high concentrations of PGE_2 and $PGF_{2\alpha}$ in the allantoic fluid and elevated mRNA expression for IL-8, TNF-α, IL-6, and IL-1β in the chorioallantois.[85]

Treatments to modulate inflammation in placentitis

Pentoxifylline is a theobromine derivative, non-selective phosphodiesterase inhibitor. Phosphodiesterase inhibitors can decrease uterine activity by increasing intracellular c-AMP concentrations and thus lowering Ca++ concentration. Pentoxifylline down-regulates pro-inflammatory cytokines such as TNF-α, IL-6 and IFN-γ. This drug also increases erythrocyte flexibility, fibrinolytic and tissue plasminogen activator activity and inhibits platelet adhesion.[86]

In mares, pentoxifylline has been shown to reach the allantoic fluid in normal pregnancy and in experimental placentitis models.[87] Pentoxifylline has been detected in placental and fetal tissues at foaling, confirming its ability to cross the placenta and reach the foal.[88] Additionally, a combination of altrenogest, antimicrobials and pentoxifylline (8.5 mg/kg PO BID) resulted in increased number of live foals in an induced placentitis study.[89]

Non-steroidal anti-inflammatory agents such as flunixin meglumine (1.1 mg/kg) are commonly used as part of pregnancy maintenance therapy. Support for this modality can be found in the finding of high concentrations of PGE_2 and $PGF_{2\alpha}$ in the allantoic fluid as described above.[85]

Conclusion

For the practitioner, having a basic understanding of the immunology of the reproductive tract can guide the choice and application of treatments to stimulate or suppress the immune system in attempt to establish pregnancy or coax a pregnancy to term. Optimizing immunological function can only occur once anatomical defects are corrected, uterine clearance is enhanced, and specific infections have been addressed.

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Equine subclinical endometritis caused by dormant beta-hemolytic streptococci

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Bacterial endometritis as a cause of sub/infertility in the mare has been known for almost a century.[1] Over the years reproductive efficiency has increased due to improved management and treatment protocols and in well-managed breeding farms the number of open mares by the end of the breeding season is expected to be less than 10%.[2] Despite these improvements, and in some settings routine use of antibiotics, infectious endometritis maintains to be a significant problem in the mare.

Beta-hemolytic streptococci (beta strep), predominantly *Streptococcus* equi subspecies *zooepidemicus* (*Strep zoo*) and *E. coli* are the most commonly isolated pathogens from the uterus of the mare, accounting for approximately 80% of cases.[3-5] Despite no reports of bacterial resistance and high *in vitro* sensitivity to the most commonly used intra-uterine antibiotics, beta-strep infections have been identified as the uterine infection correlating with the lowest fertility in well-managed Thoroughbred mares.[6]

Inspired by the work of Jesper Nielsen demonstrating improved diagnostic sensitivity and specificity when using the uterine biopsy as a diagnostic tool compared to the swab,[4] we decided to investigate the localization of *Strep zoo* in the endometrium of different types of infected mares including experimentally infected and chronically infected mares. In the experimentally infected mares (young healthy research mares, which all cultured negative prior bacterial challenge), *Strep zoo* could be found only on the endometrial surface following uterine infusion, whereas no bacteria could be re-isolated 48 h after bacterial challenge. In the chronically infected mares (mares with a history of repeated uterine infections) *Strep zoo* was consistently found to have a multifocal distribution, located within the endometrium, thus markedly different from the experimentally infected mares. In some mares, *Strep zoo* could be visualized several millimeters into the endometrium. These findings support previous notions by Nielsen who found a significantly higher proportion of endometrial biopsies culture positive when compared to endometrial swabs from the same mare[4].

Despite a monoclonal genetic backbone, bacteria can be phenotypically heterogeneous.[7] This apparent survival mechanism was suggested in the early days of antibiotics, were it was demonstrated that despite full sensitivity to the antibiotic tested, a small fraction of the monoclonal bacteria was able to survive.[7] These cells were accordingly named persisters, as they did not grow nor die in the presence of microbicidal antibiotics.[7,8] One suggested survival mechanism used by persisters is to enter a non-dividing, or dormant state.[9] An example of such a mechanism is the ability to shut down protein synthesis and DNA replication and by doing so eliminating the mechanism of action of the antibiotic present.[10] Within the group of persisters toxin-antitoxin systems are often used to regulate metabolic activity.[8,11] Essentially, the toxin inhibits basic cellular processes, such as DNA replication or protein synthesis, whereas the anti-toxin reverses this effect. Hence, the bacterial activity level is governed by the toxin/anti-toxin equilibrium.[12]

Based on previous reports and our own clinical observations on intensely managed barren mares unable to get in foal, in spite of repeated antibiotic treatments, inability to isolate bacteria or other explanatory factors stimulated further research in this area.

Resuscitating promoting factors are molecules demonstrated to override the toxin effect mentioned above and induce active growth of otherwise inactive/dormant bacteria.[13] We recently discovered bActivate* (proprietary substance) that acts a resuscitating factor, which upon uterine instillation in subclinically infected mares induced growth of beta strep in a high proportion of mares.[14] A vehicle-control study in which problem mares were infused with either bActivate or phosphate buffered saline (vehicle), supported that growth of resident streptococci and not contamination was induced by bActivate.[15] In other words bActivate is a growth medium capable of re-activating dormant streptococci residing deep in the subclinically infected endometrium. Based on this assumption we initiated a clinical study aiming at demonstrating that subclinically infected (culture-negative) barren mares instilled with bActivate and, if strep positive, treated with systemic and intrauterine antibiotics, would have a better treatment success. The study included a group of client owned subfertile barren Thoroughbred mares identified by the theriogenology group of Hagyard, Davidson and McGee in a project led by Dr. Kristina Lu. The majority of mares had a

history of repeated uterine infections and treatments. To enter the study mares had to be barren for a minimum of three cycles, despite breeding to stallions with proven fertility and high quality veterinary management. A uterine culture was collected before and 24 hours after uterine instillation of bActivate, and only culture positive mares were treated with uterine lavage, with or without mucolytics and local and systemic antibiotics. A total of 64 problem mares were included and subsequently bred. Pregnancy was established in 53 (83%) mares. Of the 53 pregnant mares, seven were lost for follow-up and a live foal was produced by 32 mares (foaling rate 70%). In this selected group of problem mares active growth of beta strep was induced in 30/64 (47%), underlining the high frequency of subclinical endometrial beta strep infections. When evaluating a group of mares not selected by fertility or history of repeated infections, only one out of twenty was found to be activation positive (unpublished data), demonstrating a strong correlation between reduced fertility and chronic dormant uterine beta strep infections.

As no placebo group was included in the clinical study, it is not possible to determine whether fertility was significantly increased following activation and treatment. Historic data demonstrate that expected fertility in this group of mares is reduced compared to the general population (foaling rate 15-50% vs. 80-85%[16]). These data show that activation and subsequent antimicrobial treatment likely improves foaling rate, almost to the level of the normal fertile population.

Activation of dormant streptococci has made it possible to diagnose subclinical chronically infected mares in the field using standard diagnostic methods. If mares are strictly selected based on fertility and reproductive history, a group of high-risk mares can be identified in which improved treatment outcome and fertility following activation and treatment can be expected.

Further research in the chronic dormantly infected mare and in the microbiology laboratory will increase knowledge of this important disease in the barren mare.

Keywords: Beta-hemolytic streptococci, mare, endometritis, dormancy, subfertility

*bActivate is produced by Bojesen and Petersen Biotech, (www.bactivate.eu)

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Bacterial endometritis: a focus on biofilms

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Abstract

Treatment of chronic bacterial endometritis with antimicrobials is often unsuccessful. Bacteria have developed multiple mechanisms of antimicrobial resistance, including the production of biofilm. Biofilms are an extracellular matrix produced by a community of bacteria that provide antimicrobial resistance through prevention of antibiotic diffusion into the community of bacteria, a decrease in the metabolism of bacteria that increase their resistance to antimicrobial agents, and ultimately cultivate a population of 'persister cells' that are multi-drug resistant. Additionally biofilms prevent recognition of the infection by the host immune system by altering the movement and function of white blood cells, and preventing antibodies from binding to bacteria. Treatment with buffered chelators or hydrogen peroxide is unable to routinely disrupt *in vitro* preformed biofilms.

Keywords: Equine endometritis, chronic, biofilm

Introduction

Most encounters between bacteria and the equine endometrium lead to an acute period of subclinical infection and occasionally clinical symptoms. Following an acute infection in the majority of mares the invading bacteria will be eliminated and the infection resolved. However, in a minority of cases, small numbers of bacteria survive and cause persistent infections that can be difficult to eliminate. The development of acute and chronic cases of endometritis is the result of deficiencies in the mare's ability to eliminate an infection and the causitive bacteria's unique pathogenic properties.

The mare's uterine defense mechanisms to bacterial consist of physical, immunological, and mechanical barriers. Bacteria utilize numerous methods to survive degradation by the host immune system and antibiotic therapy. One survival tool utilized by bacteria is the production of a biofilm. Biofilms allow bacteria to be unrecognized by the host immune system, prevent exposure to antibiotics, and allow for exchange of genetic material leading to antibiotic resistance.

The purpose of this review is to describe how alterations to host defenses in combination with the pathogenicity of bacteria result in chronic cases of bacterial endometritis.

Pathophysiology

Host defense mechanisms

The mare has three main defense mechanisms to prevent bacterial infections in the uterus, physical barriers of the reproductive tract, the innate immune system, and mechanical uterine clearance. The physical barriers include the vulva, vagino-vestibular sphincter, and cervix. These barriers prevent feces, air and environmental pathogens from reaching the uterus. A reduction in the pathogenicity and quantity of bacteria occurs from the vulva to the cervix.[1] Any disturbance in conformation of the reproductive tract will increase the likelihood of bacteria entering the uterus. Consequently, this results in a decrease in pregnancy rates. Once bacteria have reached the uterus the mare's innate immune system is activated.

The presence of bacteria within the uterine lumen results in a rapid influx of neutrophils, immunoglobulins, and serum proteins.[2,3] This binding of complement and opsonins to bacteria greatly increase the ability and rate at which neutrophils phagocytize bacteria.[4] Neutrophils from susceptible mares have reduced in vitro ability to phagocytize bacteria as compared to resistant mares.[5,6] The inflammation associated the innate immune system results in fluid production into the uterine lumen.

The final defense mechanism against bacterial endometritis is mechanical uterine clearance of bacteria and inflammatory products. Several studies have shown that mares susceptible to uterine infections have decreased clearance of uterine fluid as compared to resistant mares.[7-10] After intrauterine

inoculation with bacteria susceptible and resistant mares have similar uterine myometrial contractions for six to eight hours after inoculation, but depresses in susceptible mares after eight hours.[11] Failure to clear bacteria and inflammatory products from the uterus, results in continued activation of the innate immune system. Resulting in a further increase in inflammatory cells, immunoglobulins, and serum proteins reaching the uterus that continue to activate the innate immune system.

A single alteration to any of the defense mechanisms of a mare may allow for colonization of the uterus with a bacterial pathogen leading to a chronic infection.

Bacterial lifestyle

Bacterial are capable of living in two different lifestyles planktonic or biofilm states. Planktonic bacteria are single bacterial cells free flowing in suspension. Bacteria in this lifestyle utilize available nutrients for procreation. These individual cells are relatively susceptible to recognition and degradation by the host immune system, susceptible to changes in environment (desiccation, lack of nutrients, etc), and sensitivity to antibiotics. However, the planktonic cell paradigm does not accurately reflect the growth of bacteria in nature that are typically associated with a biofilm.[12-14]

In the last several decades the biofilm state has been considered to be the more prevalent lifestyle with ~99% of the overall world bacterial biomass living in a biofilm. In natural environments these biofilms are invariably a multispecies microbial community harboring bacteria that stay and leave with purpose, share their genetic material at high rates and fill distinct niches within the biofilm.

The first step in biofilm formation is migration and adherence to a surface. This is typically performed through the use of flagella and type IV pili in *E. coli, P. aeruginosa,* and *K. pneumonia.*[15-17] *Strep. equi* subsp. *zooepidemicus* are non-motile and rely on movement from environmental or host factors. Individual bacteria will migrate (if capable) until other bacteria (same species or other) are encountered and micro-colonies start to form. At this point planktonic and biofilm lifestyles start to diverge, genes associated with flagella are down-regulated and genes associated with polysaccharide production increase.[18] This exopolysaccaride matrix forms the scaffold for the biofilm community.

As the community of bacteria grows in size the environment within the biofilm because heterogenous with higher concentrations of oxygen and a more neutral pH on the outside of the biofilm as compared to the core which is relatively low in available oxygen with a slightly acidic pH.[19,20] Bacteria are not organized randomly distributed within a biofilm but rather organized to best meet the needs of individual and the group.

Intercellular communication or quorum sensing is carried out through the production of bacterial products that are able to diffuse away from one cells and enter another cell.[19,21-23] Signaling between cells is critical in the development of a viable biofilm and in reacting to outside environmental stress.

One of the biggest advantages of biofilm living is the ability to acquire transmissible, genetic elements at accelerated rates. Conjugation occurs naturally among bacteria but appears to be accelerated when bacteria are in a biofilm lifestyle. This allows for the rapid horizontal transfer of genetic material making a biofilm a perfect milieu for emergence of new pathogens by acquisition of antibiotic resistance, virulence factors and environmental survival capabilities.[24,25]

Clinically biofilms can cause significant difficulty for clinician to eliminate these chronic infections once established. Bacteria within a biofilm are protected from the host immune system as white blood cells have reduced ability for movement and function, and the thick layer of exopolysaccaride (EPS) prevents antibodies from reaching bacteria deep within the biofilm. Biofilms protect bacteria from antibiotics by providing a diffusion barrier that decreases the amount of antibiotics that reach the protected bacterial colonies and creates a microenvironment that slows down the metabolism and therefore the replication rate of bacteria, which also makes them more resistant to antimicrobial agents. Ultimately, biofilms are associated with development and maintenance of subpopulations of 'persister cells'.

As antimicrobial agents come in contact with the biofilm, the agents must traverse through a layer of thick EPS, DNA, RNA, lipids and proteins in order to reach bacteria buried deep within this protective barrier.[26] Bacteria in the outer region may be killed, but a decrease in the level of antibiotics reaching the inner layer bacteria contributes to the formation of a nidus for chronic infection.

The thick layer of EPS found in biofilms not only prevents antibiotics from penetrating, but limits the diffusion of oxygen and nutrients.[27] Oxygen and nutrient deprivation consequently results in a decrease in metabolic rate as compared to planktonic or free individual bacteria.[28,29] This reduction in metabolic rate provides additional antimicrobial resistance as antibiotics typically only act upon rapidly multiplying bacteria.[30]

A popular theory currently is that growth of bacteria in biofilms produces 'persister cells'. These cells are unique in that they do not appear to grow and are highly multi-drug resistant to a wide variety antimicrobials.[31] Further work is warranted to understand the role of 'persister cells' in chronic infections and biofilms.

The innate factors of antimicrobial resistance in bacterial biofilms have led to significant challenges in human medicine. It is estimated that 65% of nosocomial infections are associated with biofilms,[32] and that treatments for biofilm based infections cost >$1 billion annually.[33-35] In equine medicine, we have just started investigating the role of biofilms in chronic infections.

It has been proposed that biofilms play an important role in chronic infections in the horse. LeBlanc and Causey have suggested that chronic uterine infections resistant to antimicrobials may be due to biofilm production.[36,37]

Evaluation of bacteria isolated from the equine uterus suggests that the majority of isolates of *Streptococcus equi* subsp. *zooepidemicus, Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumonia* are capable of producing a biofilm *in vitro* (Ferris 2014, unpublished). However, to date *in vivo* biofilm production and identification has not occurred in the endometrium of the mare.. Unfortunately, no clinical diagnostic tests are available for the detection of a biofilm related infection. In human medicine a biofilm is suspected if appropriate antibiotic therapy is administered and the infection is unable to be eliminated.[38]

Treatment options

Bacteria residing in a biofilm can be up 1000 times more resistant to treatment with antibiotics as compared to free-living (planktonic) bacteria.[39-41] The administration of antibiotics has been unable to eliminate chronic infections suspected of involving a biofilm in both human and veterinary medicine.

Work in other species has shown that buffered chelating agents (tris-EDTA) may potentiate antimicrobials and break up biofilms. Gray et al showed that EDTA disrupted the lipopolysaccharide membrane of *Pseudomonas aeruginosa* through the binding of heavy metals. A concentration dependent effect was observed on solubilizing of the carbohydrates and phosphorus present in the cell wall.[42] Essentially the EDTA is able to 'poke holes' in the bacterial plasma membrane increasing the cell permeability potentially making the organism more susceptible to antibiotic therapy. First generation tris-EDTA (0.5 M tris tromethamine and 3.5 M ethylene-diaminetetra acetic acid) has been shown to kill and/or decrease survivability of *Pseudomonas aeruginosa* isolated from the equine uterus.[43]

For buffered chelating agents to have an effect they must come in contact with the bacterial cell wall and stay in contact with the bacterial cells wall to maintain effectiveness. It has been recommended to increase the infusion size to 200 to 500 mls to allow for dispersion completely through the uterus.[36] Combination of tris-EDTA and antibiotics (amikacin, ticarcillin with clavulonic acid, and ceftiofur) decreases the minimal inhibitory concentration over treatment with tris-EDTA or antibiotics alone. (Ferris 2014, unpublished).

Tris-EDTA has been shown to induce dispersal and killing of preformed biofilms in laboratory strains of *P. aeruginosa.*[44] However tris-EDTA has been unable to consistently disrupt preformed biofilm from clinical strains of *P. aeruginosa* isolated from the equine uterus using an *in vitro* model (Ferris 2014, unpublished).

Hydrogen peroxide is a common anti-septic utilized in human and veterinary medicine to non-specifically cause lysis of bacteria.[45] In equine reproduction a common concentration of 1% hydrogen peroxide has been proposed to be beneficial in chronic cases of endometritis.[46] Using an *in vitro* system to evaluate *P. aeruginosa* preformed biofilms 1% hydrogen peroxide was capable of disrupting the biofilm in 50% of cases. (Ferris 2014, unpublished).

Conclusion

Development of chronic infections is dependent upon a decrease in host susceptibility and the pathogenicity of causative bacteria. If a biofilm is associated with these chronic infections it may be difficult to appropriately diagnose the infection and even if diagnosed treatment failure is common. Unfortunately, when a biofilm is suspected in cases of chronic endometritis classically recommended therapies are not efficacious at disrupting pre-formed biofilms. Further work is needed to investigate the effectiveness of various agents utilized in human medicine to disrupt biofilms produced by the bacteria that cause endometritis in the mare.

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Equine viral arteritis – an update

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Abstract

Equine viral arteritis is a highly contagious respiratory and reproductive disease of equids whose etiology was defined in the early 1950s. The virus was named equine arteritis virus (EAV) and the disease equine viral arteritis (EVA) because of the distinctive vascular lesions found on histopathological examination of acutely infected horses. There has been significant recent progress in understanding the molecular biology of EAV and the pathogenesis of its infection in horses. In particular, the use of contemporary recombinant DNA (e.g. reverse genetics) and genomic techniques has generated significant novel information regarding the basic molecular biology of the virus and host susceptibility. Therefore, the objective of this review is to summarize the current understanding of EVA including various risk factors, viral factors and the influence of host factors such as genetics on disease susceptibility and host immune response, as well as advances in diagnostics, potential treatment and prevention strategies.

Keywords: Equine, virus, stallion, infection, EAV, EVA, persistent infection, prevention, treatment

Introduction

Equine arteritis virus infection is highly species-specific and exclusively limited to members of the family *Equidae*, which includes horses, donkeys, mules and zebras.[1,2] Equine arteritis virus has been isolated from donkeys in South Africa[3] and Chile (Timoney and Balasuriya, unpublished data, 2014) and the viral nucleic acid has been demonstrated in an aborted alpaca fetus by reverse transcription-polymerase chain reaction (RT-PCR).[4] Following the 1984 EVA outbreak in Kentucky, it was discovered that persistently infected stallions serve as the natural reservoir of the virus within the equine population.[1,2,5,6]

A substantial percentage of all infections are subclinical or asymptomatic, especially those that occur in mares after being bred to persistently infected stallions. However, natural outbreaks of EVA are characterized by one or more of the following outcomes: abortion of pregnant mares; fulminant infection of neonates leading to severe interstitial pneumonia or enteritis; respiratory and systemic illness in adult horses and persistent infection of stallions. Clinical cases of EVA typically have an incubation period of 2 to 14 days (6 to 8 days following venereal exposure) with the most consistent clinical features of EAV being pyrexia and leukopenia. Clinical signs vary considerably among individual horses and among outbreaks and often mimic other respiratory and non-respiratory diseases. Typical cases of the disease present with all or any combination of the following signs: fever up to 41°C (105.8°F) of 2-9 day duration, anorexia, serous nasal discharge, conjunctivitis, urticaria and leukopenia; edema of the lower limbs, scrotum, prepuce, eyelids, ventral body wall and udder; abortion in the mare and fatal interstitial pneumonia or pneumoenteritis in young foals. Some 10 to 70% of stallions acutely infected with EAV subsequently become carriers and constantly shed the virus in semen.[1] The EAV carrier state can be short or convalescent (lasts only a few weeks after clinical recovery), intermediate (3 to 7 months) or long-term or chronic (lasts for years and even the entire life). The mechanism of viral persistence in the male reproductive tract is not clear. However, persistence of EAV in stallion is testosterone-dependent.[7,8]

In all viral infections, there are associated risk factors, environmental, viral and host specific. Risk factors associated with EAV transmission are summarized in Figure 1, with EAV being transmitted among horses in five major ways:[3,5]

- Respiratory: primary route of transmission in acute cases of infection, common at racetracks, shows and sales.
- Venereal: virus shed in the semen of a carrier stallion (cooled or frozen semen can be infectious).
- Other bodily secretions: urine, feces, etc.

- In-utero: virus crosses the placenta from an acutely infected mare to her unborn foal.
- Indirect contamination: tack and/or equipment shared among horses. Furthermore, there is a high risk of EVA being transferred indirectly via personnel and fomites. Special care should be taken when handling semen in laboratories prior to insemination or preparation for shipping.

Disease is the result of the interaction between the infectious agent (e.g. virus), the host factors and the environmental factors. The interaction between virus-infected cells and host defense systems determines the disease outcome. These interactions are a complex, multifactorial process, which has not been fully characterized for EVA. Viral factors responsible for the virulence phenotype of some strains have been identified by using reverse genetics and were reviewed previously.[9-11] The virulence determinants of EAV have been mapped to genes encoding both nonstructural (nsp1, nsp2, nsp7 and nsp10) and structural proteins[12] (GP2, GP4, GP5 and M). However, it appears that the most important virulence determinants of EAV are located in the structural protein genes of the virus. The interaction among the GP2, GP3, GP4, GP5 and M envelope proteins plays a major role in determining the tropism of EAV for CD14$^+$ monocytes, whereas tropism for CD3$^+$ T lymphocytes is determined by the GP2, GP4, GP5 and M envelope proteins but not the GP3 protein.[13] Using an *in vitro* cell culture model of persistent EAV infection, it has been shown that combined amino acid substitutions in E, GP2, GP3, and GP4 proteins or a single amino acid substitution in the GP5 protein can facilitate persistent EAV infection in HeLa cells. However, the viral protein(s) involved in establishment of persistent infection in the stallion's reproductive tract are yet to be definitively identified. In summary, the virulence determinants of EAV appear to be complex and to involve multiple genes encoding both envelope and nonstructural proteins.

All horses appear to be susceptible to EAV infection; however, there are considerable differences in seroprevalence between different breeds of horses. In the United States, some 70-90% of adult Standardbred horses are seropositive for EAV, as compared to only 1-3% of the Thoroughbred population. Similarly, a high percentage of European Warmblood horses are seropositive for EAV. While breed-specific differences might reflect different cultural and management factors, it has recently been demonstrated that an inherent genetically conferred resistance or susceptibility of CD3$^+$ T cells to *in vitro* infection with the virulent Bucyrus strain of EAV. Specifically, based on the *in vitro* susceptibility of CD3$^+$ T lymphocytes to EAV infection, horses can be segregated into susceptible and resistant phenotypic groups (Figure 2) as described by Go et al.[14] Genome-wide association studies (GWAS) of these horses identified a common, genetically dominant haplotype associated with the *in vitro* susceptible phenotype in the region of ECA11 (49,572,804-49,643,932).[14] Biological pathway analysis identified a variety of cellular genes within this region of ECA11 that encode proteins associated with virus attachment and entry, cytoskeletal organization and NF-κB pathways. These cellular proteins are likely to be important in the pathogenesis of EVA infection in horses.[14] Additionally, host cellular factors that are involved in formation of the replication complex and other membrane structures during virus replication may play a role in pathogenesis of EVA.[9]

Experimental EAV infection of horses with either the *in vitro* CD3$^+$ T cell susceptibility or resistant phenotype showed a significant difference between the two groups in terms of proinflammatory and immunomodulatory cytokine mRNA expression. Of considerable importance, clinical signs of disease were enhanced in horses possessing the *in vitro* CD3$^+$ T cell resistant phenotype.[14] These studies provide direct evidence for a correlation in individual horses between their genotype and the extent of viral replication, occurrence and severity of clinical signs, and cytokine gene expression after experimental EAV infection. Furthermore, CD3$^+$ T lymphocytes from EAV persistently infected (carrier) stallions were susceptible to *in vitro* EAV infection,[15] whereas stallions that did not become long-term carriers after infection with EAV did not possess the CD3$^+$ T lymphocyte-susceptible phenotype (Figure 2). When considered together, these data suggest that stallions with EAV susceptible CD3$^+$ T lymphocytes are at a higher risk of becoming persistently infected as compared to stallions that lack this phenotype and GWAS analysis confirmed that five of seven proven carrier stallions had the dominant ECA11 haplotype previously associated with the CD3$^+$ T lymphocyte susceptible phenotype.[9] Further studies on genes located in ECA11 will enhance our understanding of the pathogenesis of EAV infection

and variation in susceptibility or resistance to establishment of the carrier state in stallions and will hopefully lead to the identification of a putative EAV cell receptor(s).

Diagnosis

The differential diagnosis of EVA includes other acute viral respiratory tract infections common at racetracks, shows, sales such as:

- Equine herpesviruses 1 and 4
- Equine influenza virus
- Equine rhinitis A and B viruses
- Equine adenovirus and Getah virus
- Also may include equine infectious anemia, African horse sickness, purpura hemorrhagica and urticaria and toxicosis due to hoary alyssum (*Berteroa incana*)

Appropriate samples for laboratory diagnosis of EVA include:

- Nasopharyngeal swab or nasal washing/swab
- Conjunctival swab
- Semen (sperm rich fraction of the ejaculate)
- Abortions – fetal fluids, placenta, lung, spleen and lymphoid tissue
- Whole blood (EDTA or citrated)
- Paired serum samples (21 to 28 days apart)

Laboratory diagnosis of EVA is currently based on virus isolation (VI) in cell culture or demonstration of viral nucleic acids by reverse-transcription polymerase chain reaction (standard RT-PCR, RT-nested PCR or real-time RT-PCR [rRT-PCR]) in clinical specimens. Serologic diagnosis of EVA is based on the virus neutralization test (VNT) and demonstration of rising neutralizing antibody titers (4-fold or greater) in paired serum samples. The VI and VNT are the current World Organization for Animal Health (OIE) prescribed standard tests for EVA (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals). Although VI is currently the OIE-approved gold standard for the detection of EAV in semen and is the prescribed test for international trade, it has been demonstrated that at least one published rRT-PCR assay has equal or even higher sensitivity than VI for the detection of EAV nucleic acid in semen samples from carrier stallions.[16,17] Very clearly, rRT-PCR has significant advantages over VI in terms of reproducibility between laboratories, ease and speed of completion and cost; thus it is logical that the most accurate rRT-PCR assay would replace VI as the prescribed test for international trade. Similarly, the VNT remains the OIE prescribed regulatory test for serologic detection of EAV infection of the horse, despite the fact that this assay is expensive, labor-intensive and time-consuming to perform. Furthermore, results of the VNT can vary markedly among laboratories when insufficient attention is paid to standardization of both test reagents and procedures; therefore, there is an urgent need to develop a highly specific and sensitive ELISA or microsphere immunoassay (Luminex) for the detection of EAV antibodies in serum samples. Incorporation of the immunogenic nonstructural proteins of EAV in such an assay is likely to improve both its sensitivity and specificity. Recently, a new commercial competitive ELISA (cELISA; VMRD Pullman, WA) has become available in the market.[18] Histopathologic examination coupled with immunohistochemical (avidin-biotin complex [ABC] immunoperoxidase) staining is also useful for the detection of viral antigens in formalin-fixed paraffin-embedded samples, as well as in frozen tissue sections.

Treatment

- Treatment of EVA consists of supportive care until the end of the acute phase of disease. Adequate rest should be provided, particularly to breeding stallions and to horses in training.
- Use of systemic antibiotics is unrewarding and usually contraindicated, except in pneumonitic foals at risk of secondary bacterial infections.
- Repeated vaccination has no impact on viral clearance in persistently infected stallions.

- Antagonists of gonadotropin-releasing hormone (GnRH) and GnRH vaccinations have been used to eliminate persistent infection and viral shedding with various levels of success. However, treatment strategies are still at the experimental level and require further evaluation.
- Transient suppression of testosterone production in carrier stallions may be a therapeutic alternative in the elimination of EAV infection.[19,20] There are preliminary data to support that the use of GnRH vaccines or antagonists can temporarily limit the shedding of the virus in the semen of carrier stallions.[20] Fortier et al reported that daily treatment with a GnRH antagonist for 35-37 days led to a temporary elimination of virus from the semen of 5/5 treated stallions.[19] Of those 5 treated stallions, 3 subsequently resumed viral shedding after treatment after 14, 28 and 42 days, respectively, and two remained negative at 114 days. However, because 2/4 control stallions also ceased viral shedding during the experimental period, the authors were not able to prove that the resolution in the first group was an effect of the treatment. Similarly, Burger et al[20,21] reported the cessation of EAV shedding by a stallion in which testosterone production was suppressed for 5 months by vaccination against GnRH. It was not possible to exclude the likelihood of viral shedding had not also spontaneously ceased. Nonetheless, antagonists of GnRH and GnRH vaccinations have been used to attempt to eliminate persistent infection and viral shedding in stallions with varied levels of success. However, no conclusive therapies to eliminate the EAV carrier state in stallions have been developed to date.

Outcome and prognosis

- 10 to 70% of exposed stallions become persistently infected with EAV and shed virus in their semen, sometimes for life.
- Persistently infected stallions are asymptomatic and may shed a large amount of virus in their semen.
- The virus can survive semen processing for cooled-shipment and cryopreservation, thus remaining infectious and readily transmitted to naïve mares at the time of insemination.
- With the exception of persistently infected stallions, there are no long-term effects after resolution of EAV infection in mares, geldings and foals (<6 months). Immunity is long-lasting and protective.
- Abortion in pregnant mares (3 months to over 10 months of gestation) is not preceded by premonitory signs.
- Congenital infection of late term fetuses leads to weak foals with interstitial pneumonia.
- Older foals acutely infected with EAV may develop a secondary bacterial pneumonia. Prophylactic administration of antibiotics may be indicated in these cases.

Prevention

For all venereal diseases in the horse, biosecurity measures are paramount. Horses and/or semen arriving without appropriate health certification should not be unloaded or used for breeding. Before semen is collected and shipped, appropriate health parameters must be tested and certifying paperwork must accompany the shipments.

- Effective strategies for control and prevention include:
  - Biosecurity: a major factor in determining success of any control program is minimizing or eliminating direct or indirect contact of susceptible horses with the secretions and excretions of EAV-infected animals.
    - Determine serologic and virologic status of all stallions contributing sperm to both cooled-shipped and cryopreserved semen A.I. programs.
    - Establish artificial insemination industry standards.
  - Preventing the establishment of the carrier state in stallions and post-pubertal colts will eliminate the disease.
    - Vaccinate colts after 6 months and prior to 10 months of age.[7]
- There is an effective and safe vaccine available for EVA (modified-live virus [MLV] vaccine in the US and Canada and killed virus vaccine in the European Union – Arvac® [Zoetis Animal Health Inc., Florham Park, NJ] and Artervac® [Zoetis Animal Health Inc., Kent, UK], respectively).

- The MLV vaccine has been shown to be both safe and effective for use in breeding mares, stallions, geldings, fillies, colts and early gestational pregnant mares[22] (up to 2 months before foaling) and during the immediate postpartum period.
- All sero-negative mares being bred to a persistently infected stallion should be vaccinated 21 days prior to insemination. Those previously vaccinated should be given a booster immunization prior to breeding.
- Stallions for breeding should be tested for neutralizing antibodies to EAV at least 60 days prior to breeding and all seronegative stallions must be vaccinated with the MLV vaccine. The vaccinated stallions should not be used for natural breeding 28 days post vaccination or in the case of stallions solely used for collection of semen for AI, within 14 days post vaccination.
- There is no evidence that a vaccinated stallion will develop the carrier state with the vaccine virus strain.
- Horses that travel for competitions or comingle with outside horses should be vaccinated.
- Late-term pregnant mares should not be vaccinated unless the risk of natural exposure is sufficient to offset the risk of potential complications of a modified-live virus vaccine.
- For EVA and other reportable diseases, more widespread screening of stallion populations and tighter quality control over laboratories providing diagnostics would increase detection of carrier stallions.

Client education

- Critical to prevention and control of EVA is the observance of sound practices which will reduce the risk of viral spread.
  - The reservoir is the carrier stallion, and prevention and detection of persistent infection are critical for control of disease outbreaks.
  - Specific measures aimed at minimizing the spread of virus:
    - Isolate and test new entries
    - Certify/test imported semen
    - Institute an appropriate vaccination protocol
    - Especially vaccinate all colts prior to puberty

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Figure 1: Transmission of EAV and the role of the carrier stallion in maintenance and spread of the virus

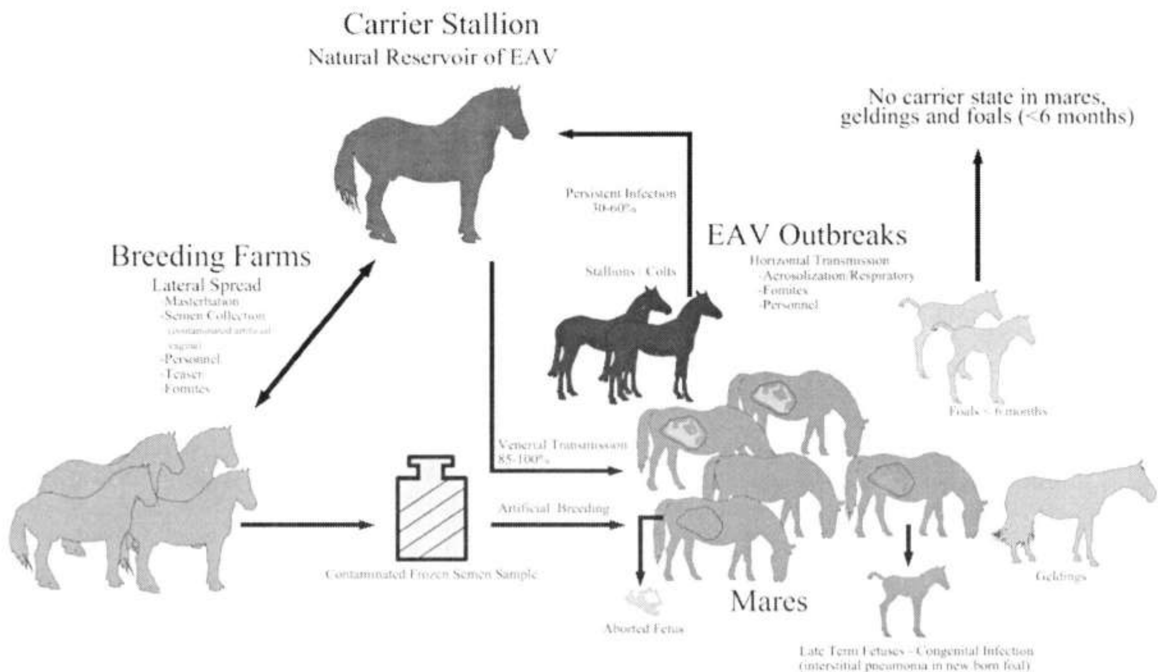
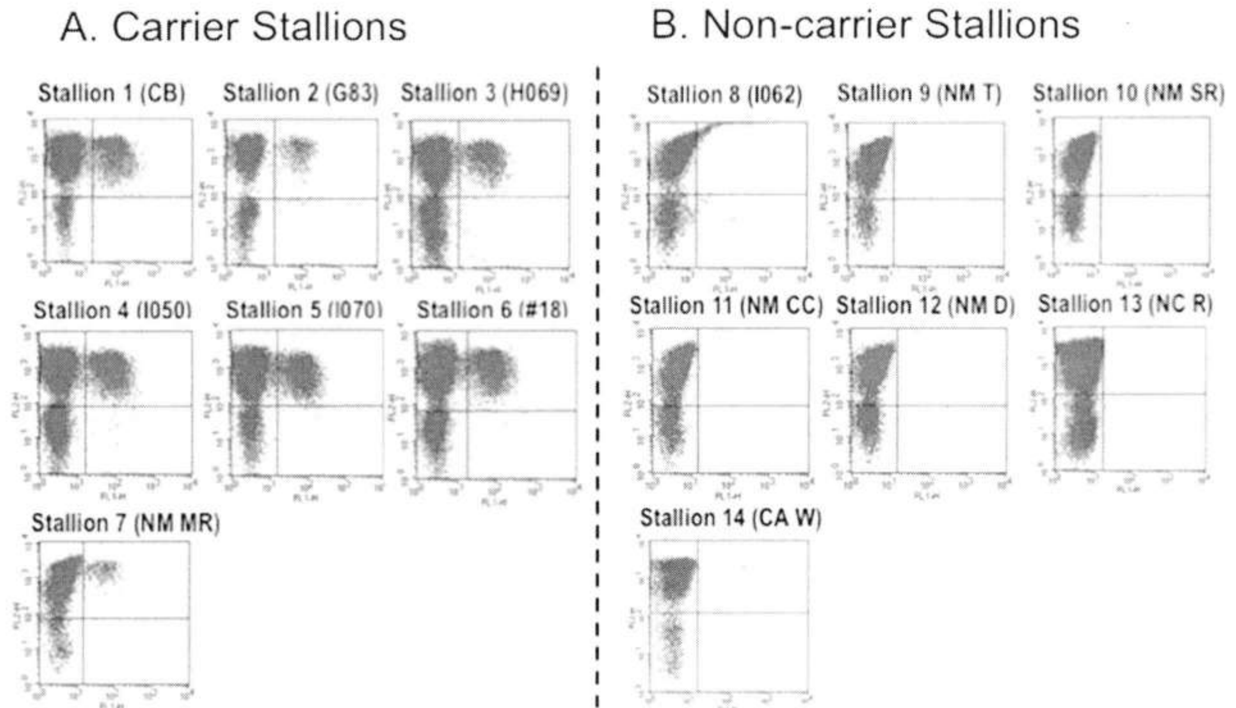


Figure 2. Dual-color immunofluorescence flow cytometry analysis of $CD3^+$ T cells from carrier and seropositive non-carrier stallions following *in vitro* infection with EAV. (A) Carrier status was confirmed by repeatedly isolating EAV from semen over an extended period of time. (B) Non-carrier stallions were uninfected but seropositive to EAV following natural infection, not vaccination. Lymphocytes from each stallion that were labeled with both anti-CD3 and anti-nsp1 (12A4) MAbs are shown in the upper right quadrant of each dot plot. Note significant double labeling of lymphocytes from the carrier stallion but not the non-carrier stallion for both CD3 and EAV after *in vitro* infection. (Adapted from Go et al., [2012] with permission; Copyright© American Society for Microbiology, [Journal of Virology, 86(22), 2012, 12407-12410 and DOI: 10.1128/JVI.01698-12]).



(Editor's note: Figures in this manuscript are available in color in the on-line version of Clinical Theriogenology.)

Use of molecular diagnostics for infectious equine pathogens

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Objectives of the presentation

- Review the basic understanding of polymerase chain reaction (PCR)
- Understanding the advantages and pitfalls of PCR
- Discuss PCR applications for respiratory, neurologic and enteric pathogens

Take home message

- Polymerase chain reaction is a nucleic acid-based amplification technique that has been described as genetic photocopying. Polymerase chain reaction allows the detection of infectious pathogens in host tissues even when only a small amount of pathogen is present.
- Polymerase chain reaction has distinct advantages as a diagnostic tool and is best used in clinical situations requiring rapid, sensitive and specific answers, which is often the case when dealing with highly infectious organisms.
- Whenever using molecular detection assays, one must keep in mind the biology of the suspected pathogen to be detected, the disease stage of the animal, the adequate specimen to be used and test to be requested. The use of a panel strategy will truly facilitate the choice of assay requested by widening the spectrum of common pathogens involved with a specific syndrome.
- Polymerase chain reaction should always be interpreted within the clinical context. If the results don't match your clinical impression, either repeat the test or investigate the laboratory's performance.

Introduction

The focus of rapid diagnosis of infectious disease of horses in the last decade has shifted from the conventional laboratory techniques of antigen detection, microscopy and culture to molecular diagnosis of infectious agents. Equine practitioners must be able to interpret the use, limitations and results of molecular diagnostic techniques as they are increasingly integrated into routine microbiology laboratory protocols. Polymerase chain reaction is the best known and most successfully implemented diagnostic molecular technology to date. It can detect slow-growing, difficult-to-cultivate or uncultivable microorganisms and can be used in situations in which clinical microbiology diagnostic procedures are inadequate, time-consuming, difficult, expensive or hazardous to laboratory staff. Inherent technical limitations of PCR are present, but they are reduced in laboratories that use standardized protocols, conduct rigid validation protocols and adhere to appropriate quality control procedures. Improvements in PCR, especially probe-based quantitative PCR (qPCR), have broadened its diagnostic capabilities in clinical infectious diseases to complement and even surpass traditional methods in some situations. Automation of all components of PCR is now possible, which will decrease the risk of generating false positive results due to contamination. The diagnostic PCR applications most relevant for equine practice are presented below along with their advantages and potential pitfalls.

Respiratory pathogens

Respiratory pathogens are often contagious, and these infections must be diagnosed rapidly in order to prevent a disease outbreak and to institute the appropriate management plan. The short turn-around time and reliability of PCR makes this technology an ideal tool for the diagnosis of respiratory pathogens.

Equine influenza is commonly diagnosed by virus isolation or detection from nasopharyngeal swabs collected from horses during the early febrile stage of the disease. Although isolation of the virus is essential to allow antigenic and genetic characterization of the strain, this technology is time-

consuming and successful isolation is, to be expected at best in about 50% of the cases. In recent years new methods for virus detection, such as antigen detection enzyme-linked immunosorbent assays (ELISA) and PCR, have been described allowing a rapid diagnosis in the acute phase of infection. Polymerase chain reaction based assays have been described for the identification of equine influenza virus directly from clinical samples with higher sensitivity than virus isolation and antigen-capture ELISA. Amplification of the single stranded RNA of equine influenza viruses is performed by reverse transcription-PCR (RT-PCR) technology, using either a one-step, nested or real-time approach. The hemagglutinin, nucleoprotein and matrix genes are the common target for these PCR assays. Unfortunately, comparison of the different PCR assays is precluded by the use of different technologies, the lack of standardization among the assays, and variation in targeted genes. Nucleotide and deduced amino-acid sequences of portions of the hemagglutinin gene are now routinely used for phylogenetic characterization of outbreak strains. Further, novel qPCR assays can be used as a viable replacement for the more traditional methods of quantifying equine influenza virus in vaccine efficacy studies. Another advantage of PCR is the ability to detect non-viable virus, a situation which may occur when nasopharyngeal samples are frozen or not adequately stored and/or shipped to a diagnostic laboratory.

Equine herpesvirus (EHV)-1 and EHV-4 are important, ubiquitous equine viral pathogens, that cause important economic losses in the equine industry. Both are double-stranded DNA α-herpesviruses that affect the equine respiratory tract and can establish life-long latent infection after primary exposure. Traditionally, virus isolation has been the gold standard for diagnosing EHV-1 and EHV-4 infections using blood and nasal secretions. Virus isolation is often hampered by the fragility of the virus, intermittent viral shedding and the interference with local antibodies. Polymerase chain reaction offers an alternative to virus isolation and has proven to be a sensitive method of detecting EHV-1/-4 in respiratory secretions, peripheral blood lymphocytes and other tissues. Many conventional PCR assays have been established to study the pathophysiology and improve the diagnosis of these viruses. Conventional one-step or nested PCR assays do have inherent risks of carry-over contamination due to post-amplification steps required to detect the PCR products. Novel molecular platforms such as the qPCR have strongly reduced the risk of contamination. Polymerase chain reaction assays used in routine diagnostics are based on the detection of viral genomic DNA and are therefore unable to distinguish between lytic, non-replicating or latent virus. Alternative molecular approaches have recently been established using the qPCR to allow discrimination between the different viral states in horses naturally infected with EHV.

Streptococcus equi subsp. *equi* (*S. equi*) infection rarely is associated with detection difficulties when using conventional culture in clinically affected horses. Culture of nasal swabs, nasal or guttural pouch washes or exudates aspirated from an abscess remains the gold standard for the detection of *S. equi*. Culture however may be unsuccessful during the incubation and early clinical phase of infection. Further, the presence of other β-hemolytic streptococci, especially *S. equi* subsp. *zooepidemicus*, may complicate interpretation of culture results. Available PCR assays are designed to detect the DNA sequence of the *S. equi* M protein (SeM) gene, the gene for the antiphagocytic M protein of *S. equi*. This gene offers enough nucleotide variations between the two *S. equi* subspecies to allow full discrimination in clinical specimens. The test can be completed in a few hours and results may be available on the same day samples are taken. One of the pitfalls of PCR has been its inability to distinguish between dead and live organisms, therefore, positive results have in the past been considered presumptive until confirmed by culture. Nowadays, the viability issue can be addressed by quantitation of the SeM gene or detection of transcriptional activity of the SeM gene at the RNA level. In several studies, PCR proved to be up to three times more sensitive than culture. Polymerase chain reaction accompanying culture on a nasal swab or guttural pouch lavage may be used in a control program to select possible carrier animals. PCR should be considered to detect asymptomatic carriers, establish the *S. equi* infection status of asymptomatic horses and determine the success of *S. equi* elimination from the guttural pouch. A particular problem in the management of strangles outbreaks is the lack of a suitable PCR assay to differentiate between wild-type and vaccine *S. equi* strains.

Rhodococcus equi is an important cause of chronic suppurative bronchopneumonia with extensive abscessation in foals three weeks to six months of age. Culture of the organism from tracheal

wash (TW) fluid currently is considered the gold standard for diagnosis. However, it can be difficult to reliably grow *R. equi* from a single TW sample, possibly because of prior antimicrobial administration or overgrowth by multiple pathogenic bacterial species. Polymerase chain reaction has been evaluated in order to increase the diagnostic sensitivity of TW fluid samples. Strains of *R. equi* isolated from sick foals uniformly contain an 85- to 90-kb plasmid that carries the gene responsible for expression of a 15- to 17-kDa antigen (vapA) of undetermined function. Environmental strains of *R. equi* not associated with disease do not contain this plasmid. Therefore, detection of the vapA gene of *R. equi* in a TW fluid sample from a foal with pneumonia can be considered diagnostic. Polymerase chain reaction should be used in conjunction with standard culture because of the possibility that multiple bacterial pathogens are present in the lower airways and the inability of PCR to determine antimicrobial sensitivity of *R. equi*. Polymerase chain reaction with its higher sensitivity and specificity may be useful to rule out *R. equi* pneumonia in culture-negative foals that have failed to improve with standard antimicrobial therapy and have clinical signs consistent with *R. equi* pneumonia. In clinical situations where the severity of the respiratory signs of the patient prevents the collection of TW fluid, feces have been shown to be a sensitive surrogate specimen for the molecular detection of *R. equi*.

Equine rhinitis A and B virus and equine arteritis virus, although less commonly associated with infectious upper respiratory tract diseases (IURD), are routinely detected via PCR. The role of EHV-2 and EHV-5 in nasal secretions of horses with IURD is still unclear. Due to their high prevalence in horse population and in order to avoid dilemmas with the interpretation of PCR results, the testing of γ-herpesviruses is at this time not recommended.

Neurologic pathogens

Although highly sensitive and specific PCR assays have been developed for the detection of viral and protozoal pathogens in the cerebrospinal fluid (CSF) of neurologic patients, these methods often are of limited value in the routine diagnosis of these diseases because viremia is often very short-lived or the pathogen has no affinity to the cells of the CSF. Consequently, pathogens are usually no longer detectable at the onset of systemic or CNS signs.

Equine protozoal myeloencephalitis (EPM), caused by the protozoal apicomplexa parasites *Sarcocystis neurona* and *Neospora hughesi*, represents one of the greatest diagnostic challenges for equine practitioners. Molecular diagnostics have also been investigated but their sensitivity was found to be low. Apparently, intact merozoites rarely enter CSF and free parasite DNA is destroyed rapidly by enzymatic action. Based on its low sensitivity, PCR testing of CSF should not be recommended for routine diagnosis of EPM. In contrast, PCR testing of neural tissue has been shown to be useful as a postmortem test.

Diagnosis of West Nile virus (WNV) encephalitis in horses currently is based on observation of compatible clinical signs and the detection of serum IgM antibody to WNV by IgM-capture ELISA. Given the non-specificity of the IgM ELISA (i.e. does not differentiate between disease and exposure) and the time required to serologically confirm WNV infection, alternative tests able to rapidly detect WNV in clinical specimens are important. RT-PCR using either a one-step, nested or real-time approach has been evaluated to investigate ante-mortem cases of suspected WNV encephalitis in horses and humans using blood. The diagnostic sensitivity of WNV RT-PCR using either serum or whole blood was very low. However, 57 to 70% of CSF samples from human beings with serologically confirmed WNV infection tested positive by qRT-PCR. The reduced ability to detect WNV in CSF or serum from equine patients with serologically confirmed WNV infection is likely due to the short-lived viremia in dead-end hosts, and emphasizes the fact that in order to detect WNV in blood or CSF, the sample should be collected early during the disease process. Investigation of the sensitivity of RT-PCR on CSF from horses with WNV encephalitis has not yet been reported.

Equine herpesvirus myeloencephalopathy (EHM) is supported by historical and clinical findings, the presence of xanthochromia and elevated total protein concentration in CSF and the laboratory detection of EHV-1 in blood and/or nasal secretions by PCR. Because affected horses can shed the virus in nasal secretions and thus represent a risk of infection for unaffected in contact horses, it is imperative

to determine the risk of shedding in a suspected horse in order to initiate an appropriate infectious disease control protocol. The dilemma as to whether the virus is in a lytic, non-replicating or latent state can be addressed by using absolute quantitation or transcriptional activity of the target gene similar to the approach used for EHV-4. Research groups have recently identified regions of variation in the genome of different EHV-1 strains (neuropathogenic versus non-neuropathogenic). A single nucleotide polymorphism at position 2254 of the DNA polymerase gene (ORF 30) has been associated with a higher risk of EHM development. Rapid PCR assays have been established to allow differentiation between neuropathogenic and non-neuropathogenic strains. However, such assays have moderate specificity, since 74 to 87% of EHV-1 stains associated with EHM have been shown to be of the neuropathogenic genotype. Therefore, these assays should be used judiciously and the results should always be interpreted in the context of clinical presentation. Further, these assays should be coupled with additional assays targeting conserved regions of the EHV-1 genome.

Gastrointestinal pathogens

The detection of equine gastrointestinal pathogens using conventional tests can be challenging because these pathogens are sometimes difficult to grow. The use of fecal material for molecular diagnostics of selected pathogens has been associated with false negative results due to the presence of inhibitory substances in the feces that can interfere with nucleic acid extraction or amplification.

Salmonella enterica can cause enterocolitis in susceptible horses; however, infection can also be present without clinical disease in 1 to 5% of healthy horses and such horses are transient subclinical shedders. Several factors, including transportation, surgery, antimicrobial treatment, changes in diet, elevated ambient temperatures and pre-existing gastrointestinal disease, have been associated with the development of clinical salmonellosis in susceptible horses. Because these factors are often common among hospitalized horses and because the contamination of the environment by subclinical shedders poses a risk to the health of hospitalized patients and personnel, the rapid identification of horses infected with *Salmonella enterica* is of considerable importance and allows implementation of appropriate infectious disease control measures.

Microbiologic culture of feces is considered the gold standard in the detection of horses shedding *Salmonella enterica*. Time to microbiological culture and positive identification of *Salmonella* from feces by clinical laboratories requires at least 48 hours. When small numbers of *Salmonella* are present in feces, enrichment steps using selective broth are required, which prolongs the detection time even further. In recent years, PCR assays have been evaluated for the detection of *Salmonella enterica* in feces from horses admitted to veterinary hospitals. Collectively, these studies have shown that significantly more fecal samples tested positive by PCR than by microbiological culture. Today's modern approach to screen environmental samples and feces is PCR testing of samples following a selective enrichment step (20 hr) coupled with conventional microbiological identification. Polymerase chain reaction has the advantage of having a very short turn-around-time and results can be available within 4-6 hours of having completed the selective enrichment step, which is still 2.5 days shorter than identification through conventional culture. However, conventional culture will remain the only diagnostic tool allowing serotyping and minimum inhibitory concentration (MIC) testing if needed.

Neorickettsia risticii is the rickettsial agent responsible for equine neorickettsiosis (EN) or Potomac horse fever, a serious enterocolitis of horses. A provisional diagnosis of EN is often based on the presence of typical clinical signs and the seasonal and geographical occurrence of the disease. A definitive diagnosis of EN, however, should be based on isolation or detection of *N. risticii* from blood or feces of infected horses. Isolation of the agent in cell culture, although possible, is time-consuming and not routinely available in many diagnostic laboratories. The development of *N. risticii*-specific PCR assays has greatly facilitated the diagnosis of EN. Nucleic acid of *N. risticii* can be detected in the blood and feces of naturally or experimentally infected horses, but the detection period does not necessarily coincide between the two sample types. Based on these results, it is recommended to analyze both blood and feces from suspected horses in order to enhance the chance of molecular detection of *N. risticii*.

An emerging equine gastrointestinal pathogen, *Lawsonia intracellularis*, has been described in young horses. This obligate intracellular bacterium is the causative agent of proliferative enteropathy (PE), a transmissible enteropathy known to affect a wide range of domestic and wild animal species. This disease has a worldwide distribution and likely is under-recognized in horses. Ante-mortem diagnosis can be challenging and is based on interpreting clinical signs, clinicopathologic results, ultrasonographic findings and excluding other causes of similar gastrointestinal findings. Currently, culture of the organism is difficult and is not routinely offered by laboratories. Diagnosis relies on serology and PCR. The combination of both tests as well as repeated fecal sampling for PCR from target animals will increase the chance of diagnosing the disease. Novel PCR assays, such as qPCR, have increased the sensitivity of molecular detection, compared to initial conventional assays. PCR has the advantage of being fast and can yield positive results in the early stage of disease, when antibodies are not yet measurable. Furthermore, the molecular assay can be used to monitor treatment success and study the epidemiology of this pathogen.

The detection of equine coronavirus (ECoV) by PCR in the feces of foals with fever and diarrhea is difficult to interpret because ECoV has also been detected in the feces of healthy foals. Healthy foals have been found to be infected mostly by ECoV in a single infection without any other coinfecting agents, whereas ECoV was found exclusively in association with other coinfecting agents in sick foals. This is in agreement with coronaviruses in other species, where the virus may not have enough pathogenic potential to cause disease, but causes local immune suppression allowing secondary infections to take place more efficiently. In adult horses ECoV causes a self-limiting disease characterized by depression, anorexia, fever and less frequently changes in fecal character and colic. More epidemiological studies are needed to better understand the impact of this emerging disease.

Conclusions

The number of commercially available PCR assays continues to expand and many molecular assays continue to be developed in the research setting. In the meanwhile, efforts should continue to increase understanding of the strengths and limitations of these new assays. Molecular diagnostic tests may enhance diagnostic capabilities, but they should be interpreted within clinical context and on the basis of individual laboratory performance. Extensive clinical research and strict adherence to guidelines for method validation are necessary to compare new molecular diagnostic techniques with existing methodologies, to validate new technology when comparable conventional techniques are unavailable, and to determine a method's clinical utility. Probe-based qPCR is an established research tool to quantify infectious agents during disease and after vaccination or therapy. Adaptations of these research applications will continue to impact testing in clinical laboratories.

Biosecurity approaches for equine reproduction

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Objectives of the presentation

- Review basic principles of biosecurity
- Discuss practical applications of biosecurity to prevent transmission of contagious pathogens

Take home message

- Biosecurity and infection control are important aspects of the day-to-day operation of any equine facility and are especially important for equine hospitals.
- It is essential that the people in charge, such as clinicians, owners, and trainers observe the implemented infection control measures.
- Leadership by example is the best way to ensure compliance of all personnel.
- In addition, educational efforts should be undertaken to make sure all workers understand the importance of biosecurity and their role in maintaining the facility as a safe place for horses and their human caregivers.

Introduction

Application of the concepts of biosecurity and bio-containment is important not only in veterinary hospitals, but also for ambulatory practices, equine breeding facilities, training facilities and other facilities that house horse populations. A passive attitude towards infection control may have detrimental consequences, including a large financial impact. Outbreaks at equine veterinary hospitals are often associated with public relations issues, such as loss of confidence and business of those who would bring their animals to the premise. Further, some contagious diseases can lead to severe disease with possible death in infected animals. This may potentially lead to litigation issues. Last but not least, outbreaks diminish morale of hospital staff and clinicians.

Basic principles of biosecurity

Properly implemented biosecurity measures may significantly decrease the risks for disease introduction and spread of infectious pathogens. Infectious disease control relies on several basic principles, which include understanding the biology of infectious pathogens and route of pathogen transmission, housing horses based on exposure risk, daily monitoring for signs associated with infectious diseases, implementing proper hygiene and cleanliness protocols, educating the horse community and having a contingency plan in place in case of an outbreak. Most of these steps will prevent and minimize exposure to infectious pathogens at an individual and population level.

Farm-based infectious disease control measures should include the segregation of horses into small groups based on age, use and gestational time. This measure may not eliminate infectious diseases in horses but, hopefully, may limit the severity of the problem by minimizing the number of affected animals. Housing at boarding facilities or horse event may represent a true challenge due to the high traffic and horse density. However, in order to minimize risk of disease outbreak, each horse should be considered at risk and handled like a single unit. Ideally such horses should be kept in individual stalls with no direct contact to other horses. The health status of such horses needs to be assessed and possibly recorded daily. Further, reducing unnecessary movement of animals and humans is an effective way to minimize spread. If equipment (grooming, cleaning and tack equipment) is shared between horses, it should be cleaned and disinfected after every individual use. Specific housing measures apply to hospitalized patients. This means that all patients are to be screened before admission to the hospital for signs of contagious disease (physical examination and accurate history). Patients should be hospitalized in dedicated stalls/barns based on their infectious status. Isolation wards should be available for patients

with confirmed and/or suspected contagious diseases. It is important to maintain hygiene of personal and facility so that hospitalized horses and their bodily fluids and excrements stay separated from other horses. It is important to monitor patients daily for the occurrence of infectious diseases through observation of clinical signs (fever, diarrhea, nasal discharge, coughing) and through strategic testing of biological samples. For example, feces may be collected for culture or polymerase chain reaction (PCR) to detect *Salmonella* spp. in high risk patients on admission and at regular intervals thereafter during the entire hospitalization time. Even with well-established infectious control protocols, it may not be possible to virtually eliminate all risks of nosocomial infections.

Probably one of the most underutilized principles of biosecurity is the daily monitoring of at-risk horses. The idea behind the assessment of daily health is to recognize early clinical signs and to take proper action to prevent disease spread. Daily monitoring by owners, trainers and care takers should include the assessment of attitude, appetite and rectal temperature. Additional signs such as nasal discharge, coughing, changes in fecal character and acute onset of neurological signs should also be recognized and reported to a health care provider.

Of all the possible measures that can be taken to reduce nosocomial and zoonotic infections, hand hygiene is perhaps the most important and cost-effective, easiest to use but also most underused measure. Hands should be washed before and after attending each individual animal. In addition to soap and water, alcohol-based hand sanitizers can be a useful adjunct to hand washing in veterinary hospitals and can provide a practical option for improving hand hygiene for ambulatory practitioners.

One additional means to prevent exposure to zoonotic pathogens and prevent transmission via contaminated hands and clothing is the use of personal protective equipment (PPE). Standard outerwear should be clean and should be changed if contamination occurs. Also dedicated clothing and footwear should be worn when working with infectious patients and in high-risk areas such as intensive care unit, isolation, foaling facility, quarantined barns, etc. Minimal PPE when working with infectious pathogens should include designated scrubs/coveralls/lab coats, disposable gloves and shoe covers/dedicated shoes/boots. Contamination of personal items such as stethoscope, thermometer, pencil, phone, pager, etc. occurs routinely when working with horses. In order to minimize exposure and transmission with infectious pathogens, one should strongly consider disinfecting all mentioned items if such items have been used while attending the patient.

Virtually all pathogens in equine facilities are associated with some organic matter, including feces, urine, saliva and sweat. Experimentally, cleaning alone has been shown to decrease the bacterial load by 90% on a concrete surface. Another 6-7% of bacteria are killed by disinfectants. There is enough convincing evidence of the necessity to clean surfaces thoroughly before disinfection. Even the best disinfectants are less effective in the presence of organic matter. Housing stalls and trailers should be cleaned and disinfected between horses. Also consider regular hosing and disinfection of aisles and high traffic areas such as wash stalls and treatment/examination rooms.

It is important to have a contingency plan in place on what to do when dealing with a potentially infectious animal. The plan should be known by all caretakers, trainers and owners at a boarding facility and by all staff and veterinarians at a veterinary hospital. Ideally, written protocols should be available and regularly reviewed and updated if needed. A logical action plan should include: general recommendations to what represents a trigger point (i.e fever, acute onset of nasal discharge, coughing, ataxia, diarrhea); isolating a sick animal (s) in previously designated areas; designate a dedicated care taker and equipment to attend the care of the sick horse; use of PPE when attending the sick horse; close or disinfect areas where the sick horse was housed or held; institute barrier nursing to prevent spread of infectious pathogens (foot bath, gloves, dedicated clothing and foot wear); contact the care provider to evaluate the horse and collect diagnostic samples; reduce overall traffic within premise and monitor horses with possible contact to the index case.

Infection control for gastrointestinal pathogens

Horses are very vulnerable to infectious enteric disorders, especially salmonellosis and clostridial infections. Several factors including stress, transportation, changes in feed, fasting, surgery, antimicrobial

use, concurrent gastrointestinal (GI) disturbances and elevated ambient temperature have been linked to an increased susceptibility to *Salmonella enterica* infection. Salmonellosis and/or clostridial infection should be considered when horses develop gastrointestinal signs (colic, diarrhea), fever and leukopenia. Further, the previous use of antimicrobials in patients developing any of the mentioned clinical signs is highly suggestive of an infectious GI disorder. Outbreaks with enteric pathogens can be devastating in any situation (farm and hospital). To determine the magnitude of the outbreak, all horses or a representative sample of resident horses should be screened for enteric pathogens using conventional microbiology and/or PCR detection. Horses testing positive for an enteric pathogen (clinical or subclinical) should always be isolated from the rest of the population to decrease the exposure risk and environmental contamination. Establish barrier nursing in the form of footbath or mats in front of the isolation unit and each stall. This will minimize the spread of pathogens from stalls to clean areas. Phenolics compounds, quaternary ammonium compounds (QAC) and peroxygens compounds have been shown to retain activity in the presence of organic matter. Phenolics and peroxygen compounds are the only disinfectant to have an activity against rotavirus. Peroxygens and high concentration of bleach (8 oz/gallon) are effective at neutralizing clostridial spores. Phenols, QAC and bleach at 4 oz/gallon are effective against *Salmonella*. Caretakers and owners should wear gloves, protective clothing (coveralls, disposable gowns) and dedicated footwear. Good hand hygiene should be instituted (faucet with warm/cold water or hand sanitizer). It is very important to control traffic and minimize contact of affected horses with the general public. Remember that enteric pathogens such as *Salmonella*, *Cryptosporidium* and *Clostridium difficile* are potential zoonotic agents and represent a greater risk for immunocompromised humans, infants, and elderly people. Instruct caretakers/owners to handle diseased horses last and to use separate equipment (cleaning equipment, tractor, hay wagon, wheelbarrow, etc). Hygiene should be maximized by regular cleaning and disinfecting. Waste from positive animals should be either removed from the premise, or composted or spread in sunlight in a place with no direct access to horses.

Infection control for respiratory pathogens

During outbreaks of respiratory disease, aerosol and droplet infection can be minimized by separating animals according to their infections status (infected, exposed versus non-exposed).

Air movement may play an important role in transmission of aerosolized virus, since viral respiratory pathogens, such as equine influenza virus (EIV), have been shown to be transmittable over a distance of 150 feet. The α-herpesviruses (EHV-1/-4) require closer contact and are generally transmitted via nose-to-nose contact or fomites. Transmission of *Streptococcus equi* subsps. *equi* usually requires direct physical contact between infected and susceptible horses but can also be transmitted via fomites (hands, shared equipment). Fortunately, outbreaks of respiratory pathogens can successfully be controlled via appropriate infectious disease control measures (separation of infected animals, cleanliness and hygiene, restrict movement and traffic, use of PPE and barrier nursing). Common infectious respiratory pathogens are susceptible to the majority of commercially available disinfectants.

Infection control for venereal diseases

Venereally transmitted diseases such as contagious equine metritis (*Taylorella equigenitalis)*, equine coital exanthema (EHV-3) and equine viral arteritis (EAV) can have a devastating impact for breeders and veterinarians involved in breeding management of mares and stallions. These diseases are highly contagious and can be transmitted by direct horse-to-horse contact, contaminated biological samples (semen), contaminated equipment and personnel. To minimize the risk of disease instruction at a breeding facility the following steps should be undertaken: examine all new horses on arrival for signs consistent with a contagious disease; determine the status of the stallion/mare for selected infections prior to use; separate new arrivals from resident horses; monitor horses daily for signs of infectious diseases; use hygienic procedures during breeding; properly clean and store breeding equipment (vaginal speculum, artificial vagina, breeding phantom); and increase cleanliness at breeding facility. In general if performed properly, artificial insemination (AI) reduces the risk of bacterial contamination of the uterus by

eliminating the contact between stallion and mare. However, viruses are generally not killed or eliminated by cooling or freezing. The venerally transmitted pathogens are susceptible to the majority of commercially available disinfectants.

Bilateral anorchia in a dog

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An 11 month old male Cavalier King Charles Spaniel was evaluated at the University of Florida Veterinary Medical Teaching Hospital for second opinion examination of apparent bilateral anorchia. Subcutaneous, inguinal, or intra-abdominal testes or vasa deferentia were not found at exploratory laparotomy performed at nine months of age. Ultrasonography at ten months did not find evidence of gonadal tissue, prostate, or os penis. The dog was otherwise apparently healthy. The owners requested that only noninvasive procedures be performed. Testes were not identified in the scrotum or inguinal area. Micropenis was suspected based upon manual palpation through the prepuce. Per rectum examination of the caudal urethra did not identify a palpable prostate gland. Abdominal ultrasonography was performed. Testes were not found. A 6.9 mm structure consistent with the prostate was identified. A hypoechoic focus of 5.3 x 2.8 mm was identified, suggesting a possible uterine remnant. Caudal to the left kidney was a heterogeneous region of 1.3 x 0.9 cm that contained anechoic oval structures of maximal size of 4.3 x 2.9 mm, interpreted as possible ovarian or lymphoid tissue. Transcutaneous examination of the prepuce showed that an os penis was absent. Computed tomography of the abdomen and pelvis was performed in order to elucidate the potential structures identified on ultrasonography. Gonads (neither ovaries nor testes), os penis, prostate and uterus were absent. A caudal abdominal vena cava bifurcation anomaly, with branching between the left and right renal veins, was found. Inguinal lymph nodes were identified. Human chorionic gonadotropin stimulation with 200 units, im, was performed. Basal concentrations of progesterone, estrogen, and testosterone were low and stimulation was not elicited at 2 h. Normal male karyotype with no chimerism was found, as was normal presence of the sex determining region (SRY) gene and amelogenin. At 17 months of age, anti-Müllerian hormone was 0 pg/ml (reference range: > 90 pg/ml in intact dogs). Repeat human chorionic gonadotropin stimulation with 1,000 units, im, testing was performed with only testosterone assayed. Initial concentration was low and stimulation at 2 or 4 h did not occur. Confirmation of bilateral anorchia was made by means of noninvasive diagnostic testing and repeat exploratory laparotomy was avoided.

Keywords: Dog, bilateral anorchia, disorders of sexual development, anti-Müllerian hormone, computed tomography

Diagnosis and management of a granulosa-theca cell tumor in a nulliparous Holstein heifer

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Although rare, granulosa-theca cell tumors (GTCT) are the most commonly reported ovarian neoplasm in the bovine. Historically, diagnosis has centered on transrectal ultrasound, histopathology, and serum hormone assays. Appearance of these tumors is variable and may be difficult to distinguish from ovarian abscesses or cysts. In the field, biopsy of the affected ovary to confirm a diagnosis may be difficult or impractical. Recently, plasma anti-Müllerian hormone (AMH) has been identified as a potentially reliable diagnostic aid in confirmation of GTCT.

In December 2012, routine transrectal reproductive ultrasound examination of a 15-month old Holstein heifer housed at the Michigan State University Dairy showed a grossly enlarged right ovary with irregular architecture and multiple cystic structures. The contralateral ovary was inactive. The AMH concentration from a pre-surgical serum sample in this case was 5.37 ng/mL. Previously published data show that normally cycling cows have a mean AMH concentration of 0.09 ng/mL and an optimal cutoff of 0.36 ng/mL for diagnosis of GTCT has been suggested. The inhibin level was 1.53 ng/mL (published data suggests a cutoff of 0.69 ng/mL for GTCT).

In January 2013, the affected ovary was removed via a standing right flank laparotomy. A 12 cm diameter ovarian mass was exteriorized and multiple transfixation ligatures were placed on the pedicle prior to transection. Histopathologic and immunohistochemical examination of the mass confirmed the diagnosis of granulosa theca cell tumor.

Following removal of the affected ovary, the heifer began cycling normally. The heifer was artificially inseminated in March 2013 and calved in December 2013. Based on the outcome of this case, we conclude that AMH and inhibin levels may be useful to practitioners as an aid in the diagnosis of GTCT. Additionally, although this surgery was performed in a teaching hospital, it could easily be performed in the field and may preserve the reproductive life of a genetically valuable animal.

Keywords: Anti-Müllerian hormone, bovine, ovarian tumor

Semen collection from a stallion with a permanent perineal urethrostomy: a case report

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A 17 year-old American Quarter Horse stallion presented for semen collection and cryopreservation. He had a history of producing 57 registered foals over a period of 12 years. The stallion recently had a permanent perineal urethrostomy (PU) following obstruction of his penile urethra with a urinary calculus. Initially an emergency temporary perineal urethrostomy had been placed and the calculus was eventually removed. Unfortunately a stricture formed at the site where the calculus had been located and the temporary PU was converted into a permanent PU. The owner wanted to continue to use the horse as a breeding stallion. The horse had excellent libido and was able to gain a normal erection. Unfortunately, the stricture prevented semen from passing down the urethra and exiting the penis in a normal manner. The goal of this case report is to describe the technique and results of a non-traditional semen collection procedure. In order to collect semen, a small plastic bag was glued over the PU site. The stallion was subsequently teased to a mare in heat until he gained an erection and then allowed to mount a breeding phantom. The penis was directed into an artificial vagina (AV) and the stallion worked the AV vigorously. Within a few thrusts the horse ejaculated normal semen out of the PU site and into the plastic bag. The semen was analyzed for gel-free volume, sperm concentration and total and progressive motility using standard techniques. Three out of four mares became pregnant after insemination with freshly collected semen using this technique. Nine additional ejaculates were subsequently obtained (Table). Spermatozoa from two of the ejaculates were processed and cryopreserved in Botu-Crio extender, with an average post-thaw total motility of 37%. In summary, semen can be collected from a stallion through a perineal urethrostomy and used to inseminate mares or cryopreserved for later use.

Table. Semen parameters from a stallion collected through a perineal urethrostomy compared to parameters from single ejaculates obtained from 5 normal stallions (mean ± std. dev.).

Parameter	Normal Stallions	Clinical Case
# Ejaculates	5	9
Gel-free volume (mls)	23.0 ± 6.7	20.8 ± 3.7
Sperm concentration (millions/ml)	429 ± 106	172.8 ± 38.3
Total number sperm in ejaculate (billions)	9.8 ± 0.6	3.27 ± 1.0
Total sperm motility (%)	85.4 ± 1.7	45.9 ± 2.9
Progressive sperm motility (%)	78.2 ± 5.1	34.3 ± 3.3

Keywords: Stallion, semen, perineal urethrostomy, collection

The canine corpus luteum expresses IGF1 and its receptor: implications in luteal function

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Introduction and objectives

In the dog, insulin-like growth factor 1 (IGF1) is associated with nutritional status and adult body size and weight, but its function in reproduction is not known. Positive correlations between intrafollicular progesterone (P4) and plasma and follicular IGF1 levels were found in bitches by Reynaud et al[1] implying a role in luteinization and steroidogenesis. We hypothesize that IGF1, acting through its receptor (IGF1R), increases luteal P4 production and corpus luteum (CL) development in the dog. The aim of the present study was to characterize time-related changes in gene expression and cellular distribution patterns of IGF1 and IGF1R in the CL during pregnancy and in diestrus of non-pregnant animals.

Materials and methods

Pregnant bitches were ovariohysterectomized 8-12 d (pre-implantation), 18-25 d (post-implantation), 35-40 d (mid-gestation) after mating (mating=d 0, performed 2 d after ovulation) and at prepartum luteolysis (n=3-5 per group). Non-pregnant dogs (not mated) were spayed 5, 15, 25, 35, 45 and 65 d after ovulation (n=4-5 per group). Luteal IGF1 and IGF1R mRNA was determined by semi-quantitative real-time (TaqMan) PCR. Immunohistochemistry using indirect avidin-biotin-immunoperoxidase procedure was performed to localize both proteins in the CL during gestation. Statistical analysis was carried out by one-way ANOVA followed by Tukey's test, and by the Kruskal-Wallis test (IBM® SPSS® Statistics for Windows, Version 19.0; Armonk, NY). Data were logarithmically transformed when needed to approach normal distribution.

Results

Similar IGF1 expression patterns were found in pregnant and non-pregnant dogs; mRNA levels decreased from the early luteal stages to prepartum ($P=0.015$) and to d 65 postovulation ($P=0.002$), respectively. IGF1R expression was up-regulated at prepartum luteolysis compared to pre- and post-implantation ($P \leq 0.008$), but did not change significantly in non-pregnant bitches. From pre-implantation through mid-gestation, strong signals of IGF1 were detected in the cytoplasm of luteal cells, which became weak prepartum. Blood vessel intima and media also showed weak positive reaction. In general, positive signals of IGF1R were more prominent in blood vessels compared to luteal cells during pregnancy. In the prepartum luteolysis group staining of steroidogenic cells was weaker than in all other stages.

Conclusion

Insulin-like growth factor 1 may be luteotropic in the early CL supporting steroidogenesis and angiogenesis, hence luteal formation and growth.

Keywords: IGF1, pregnancy, diestrus, ovary, dog

Acknowledgement

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Reference

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Accuracy of behavioral testing for pregnancy diagnosis in the dromedary camel

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Pregnant camels display a distinct posture characterized by immobilization, head elevation and tail curling when approached by a rutting male. For centuries, Bedouins have relied on this behavior for breeding management of their camel herds and identification of non-pregnant or infertile females. The objectives of the present study were to determine the sensitivity and specificity of this behavioral testing for pregnancy diagnosis and to evaluate the timing of onset of this behavior following fertile mating.

Experiment 1: A total of 157 females that were mated 25 days previously were teased to a male. Two observers recorded the teasing results for each female as either pregnant or non-pregnant based on behavior. All teased females were submitted within one day of behavioral testing to transrectal examination by ultrasonography for pregnancy diagnosis. Additionally, blood samples were taken from all females at the time of behavioral testing and serum was stored at -20°C until assayed for progesterone by radioimmunoassay.

Experiment 2: Fifty healthy multiparous females were examined daily by ultrasonography and mated to fertile males when a mature follicle (12 to 18 mm diameter), uterine tone, and uterine edema were present. Transrectal ultrasonography was performed on all mated females 7 days later for determination of ovulation and 16 days post-mating for pregnancy diagnosis. All females were teased daily by a rutting male and behavior was recorded as pregnant or not pregnant.

In experiment 1, pregnancy diagnosis by ultrasonography revealed 56.1% pregnant (n=88) and 43.9% non-pregnant (n=69) females. Sensitivity and specificity of behavioral testing compared to pregnancy diagnosis by ultrasonography were 100% and 91.3%, respectively. Six females diagnosed pregnant by behavioral testing were not pregnant on ultrasonographic examination but had either corpora lutea (n=4) or luteinized anovulatory hemorrhagic follicles (n=2). All females diagnosed pregnant by behavioral testing had a serum progesterone level >2 ng/mL.

In experiment 2, 43 of the 50 females (86%) ovulated and showed a mature corpus luteum 7 days post-mating. Of the 43 ovulating females, 39 (90.7%) were determined to be pregnant by ultrasonography on day 15 post-mating. The percentage of females that showed behavioral signs of pregnancy increased steadily to reach 100% by day 15 post-mating (day 7=2.6%; day 8=10.3%; day 9=17.9%, day 10=17.9%, day 11=17.9%; day 12=76.9%; day 13=82%; day 14=82%; day 15=100%; day 16=100%).

In conclusion, behavioral testing for pregnancy diagnosis as practiced by Bedouins is highly accurate in normal females starting from day 15 post-mating. A small percentage of false positives may occur due to persistence of luteal activity. The typical pregnancy behavior of the female camel is caused by persistently elevated progesterone levels.

Keywords: Pregnancy, behavior, camel, ovulation, progesterone

Neonatal exposure to medroxyprogesterone acetate alters canine uterine development

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A simple, permanent, non-surgical method for sterilizing dogs would benefit both animals and society. Previous work from co-investigators has shown that neonatal administration of progestins can permanently block uterine gland formation in sheep and mice, creating a uterine gland knock-out (UGKO) phenotypic model and inducing sterility in adult animals. The objective of this study was to test the hypothesis that neonatal progestin administration in the dog alters uterine development, with particular emphasis on uterine gland formation.

Seven mixed-breed female puppies were given either a time-released progestin (n=4), medroxyprogesterone acetate (MPA; 10 mg/kg IM), or saline (n=3) at 5 days of age. Dosing was repeated in the same manner when body weight tripled (average of 2.5 weeks of age). Serum MPA levels were determined at multiple time points to ensure adequate delivery of the drug. Ovariohysterectomy (OVH) was performed at seven weeks of age and uterine tissues were examined. Uterine gland development and epithelial proliferation were assessed in the following manner: tissues were stained with hematoxylin and cross-sections were examined using the Aperio® imaging system (Leica Microsystems, Inc., Buffalo Grove, IL) from which gland penetration measurements were obtained. Additionally, uterine cross sections were stained with POPO-1 to visualize cell nuclei, cytokeratin 8 (CK8) as an epithelial marker and proliferating cell nuclear antigen (PCNA) as a marker of cell proliferation. Primary antibodies were localized using fluorochrome-labeled secondary antibodies in order to produce target-specific signals at defined emission wavelengths: A490 (POPO-1), A568 (CK8), A594 (PCNA). Images were obtained using a Nuance FX multispectral imaging system (Caliper Life Sciences, Hopkinton, MA). Spectrally unmixed images were analyzed using Cell Profiler and Cell Profiler Analyst software (Broad Institute, Cambridge, MA). Quantitative data were subjected to analyses of variance. No effects of treatment on uterine gland penetration depth were identified. However, a treatment by cell-compartment interaction was detected ($P < 0.01$) for PCNA labeling index, indicating compartment-specific reductions in cell proliferation associated with MPA exposure. While uterine gland development was not inhibited overtly, MPA did reduce canine endometrial cell proliferation in both glandular and luminal epithelial compartments. Thus, strategic neonatal progestin exposure holds promise as a tool for programming canine endometrial development and function.

Keywords: Uterus, medroxyprogesterone acetate (MPA), canine, neonate, development

Prenatal exposure to an androgen agonist and antagonist alters theovine sexually dimorphic nucleus in male but not female lamb fetuses

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The ovine sexually dimorphic nucleus (oSDN), located in the medial preoptic area, is approximately two times larger in rams than in ewes. Differentiation of the oSDN occurs during gestation days (GD) 60 to 90 and the volume of the oSDN can be enlarged by exogenous androgen exposure of female sheep fetuses. This study was designed to test the hypothesis that androgen, acting through the androgen receptor (AR) is largely responsible for gender specific brain differentiation. In experiment 1, pregnant ewes received injections of the non-aromatizable androgen dihydrotestosterone propionate (DHT), flutamide (androgen receptor antagonist) or vehicle during the critical period. Fetuses were delivered at GD 135. Treatments reduced ($P<0.05$) mean oSDN volume in males but not in females. In experiment 2, to test whether androgen may orchestrate brain masculinization indirectly, we evaluated the effects of hormone treatments on the fetal hypothalamic-pituitary-gonadal (HPG) axis. Pregnant ewes were injected with DHT, flutamide or vehicle from GD 60 to 84 and fetuses were delivered on GD 85. Gene expression data and hormone concentrations were analyzed by two-way ANOVA, with posthoc comparisons made using Fisher's (LSD) test. Statistical significance for all analyses was defined as $P<0.05$. Dihydrotestosterone propionate significantly decreased fetal luteinizing hormone (LH) and testosterone, while flutamide significantly increased both hormones in male fetuses. In female fetuses, LH was significantly reduced by DHT exposure but unaffected by flutamide. Dihydrotestosterone propionate significantly decreased both pituitary gonadotropin and hypothalamic kisspeptin expression in males and females. Neuropeptide-Y expression was not altered by treatment but was expressed at a higher level in males. Our results demonstrate a paradoxical effect of exogenous androgen exposure in that oSDN volume was reduced in male fetuses but relatively unchanged in females. This is likely due to the suppression of anterior pituitary LH secretion resulting in reduced testicular testosterone secretion in DHT-treated males. These results demonstrate that AR mediates much or all of the masculinization of the oSDN in males and suggests the effects of DHT on oSDN development are secondary to negative feedback on the fetal HPG axis which may be mediated, in part, by hypothalamic kisspeptin. Collectively, our results support the concept that the HPG axis in the male fetus responds in the expected negative feedback manner to exogenous androgens and that tonic negative feedback by endogenous androgens is disrupted by flutamide. We conclude that, during the critical period of brain sexual differentiation, the fetal reproductive axis in long gestation species, such as sheep, is sufficiently developed to react to perturbations in serum androgen levels and mitigate disruptions in brain masculinization.

Keywords: Ovine sexually dimorphic nucleus, sexual differentiation, dihydrotestosterone, flutamide, androgen receptor, kisspeptin

Leukocyte esterase test for diagnosis of endometritis in dairy cows

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Endometritis is a common inflammatory uterine disease that persists beyond normal uterine involution and impairs reproductive performance. Several methods have been described to evaluate the inflammatory processes of the endometrium however, their efficiency remains controversial. An alternative method to assess endometrial inflammation is the leukocyte esterase (LE) test. Lleukocyte esterase is an enzyme normally released by neutrophils. The hypothesis is that esterase activity reflects uterine inflammation and is associated with fertility in the postpartum period. The objective of the present observational cohort study was to validate the semi-quantitative colorimetric LE-test as a cow-side test for postpartum endometritis in a farm setting. All procedures conformed to the national guideline for care and use of laboratory animals and were approved by the institution animal care committee of the University of Montreal. A sample of 17 different commercial dairy herds for a total of 569 Holstein cows was recruited for the study. All cows were systematically enrolled on an ovulation synchronization protocol (Presynch-Ovsynch) for the first insemination. Uterine samples were collected by cytobrush for endometrial cytology (CYT) and the LE test between 26 and 42 days in milk (DIM). After rolling the cytobrush on a clean glass microscope slide, the cytobrush was plunged into a 3ml glass tube containing a 1ml of 0.9% saline. The LE strip was inserted into the glass tube and the result was recorded 2 minutes after removal. Five categories based on the color of the reagent area were used according to the manufacturer recommendations. Herd records were compiled in a databank (Dossier de santé animale: DSAHR) and validated. Pregnancy diagnosis was done by transrectal palpation 35 days after artificial insemination (AI). Reproductive data of cows were collected until at least 300 DIM. Statistical analyses were performed using a mixed logistic regression (PROC GLIMMIX, SAS) with herd as a random effect and season and lactation as fixed effects to determine the association between LE and pregnancy to first AI. Based on the highest sum of sensitivity and specificity for the pregnancy status at 120 DIM, the optimal cutoffs were 7% neutrophils for cytological endometritis (CYT+) and 1+ for endometritis based on LE (LE+). The prevalence of CYT+ and LE+ were 30, and 31%, respectively. Endometritis based on LE was detrimental to first service conception risk (LE+:23%; No LE+: 38%; P=0.03). The OR for pregnancy was 1.56 (1.00 to 2.42; p=0.05) in No LE+ compared with LE+ cows. If endometritis based on LE was grouped into 3 categories (Negative LE, Slight LE and ≥LE+), Negative LE cows (OR 1.46 0.79 to 2.73; p=0.59) were not different from the ≥LE+ cows; but cows with slight LE response (OR 3.01 1.01 to 4.76; p<0.01) had higher odds of pregnancy compared with ≥LE+ cows. Similar to LE, when cows were grouped in 3 CYT categories (Negative CYT = 0%, Slight CYT = 1 to 6% and CYT+ ≥ 7% neutrophils), the Slight CYT group (OR 3.10 1.71 to 5.60; p<0.01), but not Negative CYT (OR 1.42 0.76 to 2.66; p=0.38), had higher odds of pregnancy compared with CYT+ cows. The present study shows that the LE test is associated with uterine inflammation and may be a promising tool to predict poor reproductive performance in postpartum cows.

Keywords: Dairy cows, endometritis, leukocyte esterase

Viscoelastic modifiers and hyperactivating agents as potential selectors of superior sperm for *in vitro* fertilization

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Efficiency of bovine *in vitro* fertilization is lower than that of *in vivo* fertilization (IVF) potentially due to less stringent selection of sperm. Sperm hyperactivation is essential for fertilization and in aqueous media, hyperactivated sperm swim in tight circles but in viscoelastic media, such as medium containing long-chain polyacrylamide (PA) and uterine tubal fluid, hyperactivated sperm move progressively whereas non-hyperactivated sperm are unable to traverse such media. Our objectives were to: 1) identify hyperactivating conditions that improve sperm penetration of viscoelastic media; and 2) determine whether *in vitro* fertilization (IVF) in viscoelastic media improves cleavage rate. The hypotheses were that: 1) sperm hyperactivating agents such as caffeine, procaine, and 4-aminopyridine (4-AP) or in combinations would improve sperm motility in viscoelastic media; and 2) performing IVF in viscoelastic media would improve fertilization rates. Experiment 1 tested the effects of 1 % and 1.5 % PA and hyperactivating agents compared to control (aqueous media) on sperm movement parameters measured by CASA (CEROS, Hamilton Thorne, Beverly, MA); three bulls were tested and each bull was tested in three replicates. Outcome measures were: % total motile, % progressively motile, velocity average path (VAP), velocity curvilinear (VCL), velocity straight line (VSL), straightness (STR) and linearity (LIN). Linear regression (JMP Pro 11, SAS Institute, Cary, NC) was used to determine effects of treatments on CASA outcomes controlling for bull effects. In PA, VAP, VCL and VSL were decreased ($P < 0.05$) compared with control but STR and LIN were increased ($P < 0.05$). Total and progressive motility were lower ($P < 0.005$) in 1.5% but not in 1% PA compared with control media, but linearity and straightness were increased in both concentrations of PA ($P < 0.01$). Hyperactivating agents individually were not effective but when used in combinations had additive effects and ($P < 0.05$) increased hyperactivation. In particular, 4-AP and caffeine were effective to hyperactivate sperm in viscoelastic media. In Experiment 2, an IVF trial was performed to determine if fertilization in viscoelastic media (1.5% PA) or supplemented with 4-AP in addition to caffeine or PA plus 4-AP improved cleavage rates. Bovine embryos were produced from slaughterhouse ovaries in four replicates of IVF experiments (n = 823 oocytes). Mixed logistic regression (STATA 10.1, College Station, TX) with replicate treated as a random variable revealed that both PA and 4-AP treatments decreased cleavage rates ($P < 0.001$) although there were no additive effects of the two factors (caffeine + 4-AP). In conclusion, velocity parameters of sperm were decreased in viscoelastic media but STR and LIN were increased. Both PA and 4-AP decreased cleavage rates in IVF. This may be due to either direct damage to sperm or oocyte during incubation by these factors.

Keywords: Bovine, sperm, hyperactivation, *in vitro* embryo production, viscosity

Evaluation of the effects of pre-breeding beef heifer management on mid to late gestation uteroplacental hemodynamics

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In a well-managed beef herd, heifers should represent the most valuable genetics in the herd and be an improvement over the previous generation of females. Recent economic conditions have brought forth a trend in which heifers are developed in "low input" management scenarios where they typically achieve 50-55% (as opposed to the more traditional 65-70%) of projected mature body weight at breeding. The uterine environment during gestation is known to have lifelong epigenetic effects on offspring. This is often achieved by imposing suboptimal conditions or nutrition prior to breeding or during gestation, and the effects may be translated to the developing calf by altered patterns of uteroplacental blood flow. Thus, it was hypothesized that low input heifer development protocols resulting in lightweight heifers at breeding may cause decreased uteroplacental blood flow during pregnancy compared to traditionally developed females, even when nutrition during gestation is equivalent. Therefore, the objective of the present study was to evaluate the effects of heifer development practices on uteroplacental hemodynamics during mid- to late-gestation of nulliparous beef females. To this end, crossbred beef heifers (n=15) developed on either a low input (LOW; n=6) or a conventional (CON; n=9) heifer development scheme were bred at 15 months of age. All heifers were comingled and managed on a forage based management program. Body weight (BW) was assessed every 30 d, and Doppler ultrasonography was used to assess blood flow metrics of uterine arteries on d 180, 210, and 240 of gestation. Arterial diameter (AD), blood flow (BF), resistance index (RI), and pulsatility index (PI) were evaluated for uterine arteries contralateral and ipsilateral to the conceptus, and total blood flow (TBF) was calculated as the sum of blood flow from both. Variables were analyzed using the MIXED procedure of SAS for Windows 9.3. Heifers in the LOW group had decreased BW throughout gestation ($p=0.0003$). Uterine RI and PI were not different across treatment or day. Measures of uterine BF were not different across treatment. As expected, a main effect of day was observed for BF, whereby contralateral, ipsilateral, and TBF increased as gestation progressed ($p=0.0001$, 0.0041, 0.0003, respectively). However, when adjusted for BW, an additional main effect of treatment was observed ($p=0.0079$) in which LOW heifers had increased TBF as compared to CON heifers. Correspondingly, an interaction between treatment and gestational day was observed for AD of both contralateral and ipsilateral uterine arteries ($p=0.0236$ and 0.0087 respectively), whereby AD increased as gestation progressed, and LOW heifers displayed increased AD. It was concluded that developing replacement heifers with low input management schemes does not yield compromised uteroplacental hemodynamics as compared to traditionally developed females when nutrition during gestation is adequate.

Keywords: Uterine blood flow, Doppler ultrasonography, fetal programming, heifer development

Effects of different applications of pyrethrin/pyrethroid insecticides on bull reproductive parameters

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Increasing concern in the beef industry has arisen over the use of pyrethroid and pyrethrin insecticides due to the well documented negative effects on semen quality and steroid hormone production in a variety of mammals. However, recent experiments with beef bulls demonstrated no effects on sperm motility or morphology when pyrethroid insecticides were applied at labeled doses. The objectives of the current study were to determine the effects of pyrethroid and pyrethrin sprays used in combination with pour-on and fly tags on bull sperm motility and serum testosterone concentrations. Our hypothesis was that the addition of pyrethrin and pyrethroid spray applications would have a negative effect on bull reproductive parameters. Angus x Simmental bulls (n = 23; average initial BW = 796 ± 160 kg) were blocked by source and randomly assigned to 1 of 2 treatments: 1) pour-on and fly tags (CONT; n = 12), or 2) pour-on, fly tags, premise spray and fog spray (EXP; n = 11). The CONT group was treated with Cylence pour-on (active ingredient cyfluthrin; 1%) at the labeled dose on weeks 0 and 4 and with 2 Cylence Ultra fly tags (active ingredients beta-cyfluthrin; 8% and piperonyl butoxide; 20%; Bayer Animal Health, Shawnee Mission, KS) on week 0. The EXP group was treated as CONT group, but also received Tempo premise spray (active ingredients beta-cyfluthrin, cyano and methyl 3; 11.8%; Bayer Animal Health) once a week and LD-44Z fog spray (pyrethrins; 0.5% and piperonyl butoxide; 4%; Chem-Tech, Des Moines, IA) once a day at labeled dosages. All bulls were housed in individual stalls within barns, but treatment groups were separated by aisles throughout the entire ten week experimental period. Scrotal circumferences were measured at weeks 0 and 9. Semen was collected weekly via electroejaculation. Whole blood, as a source of serum, was collected from the tail vein weekly for peripheral blood testosterone concentrations. Semen was analyzed for progressive and overall motility using computer-assisted semen analysis. All data were analyzed in MIXED procedure of SAS and repeated measures were used to test the treatment × week interactions for fertility parameters. There were no differences in initial or final scrotal circumferences ($P \geq 0.22$). There was a treatment × week interaction ($P < 0.01$) for overall motility, progressive motility, and testosterone concentrations. At week 2, overall motility was greater ($P = 0.05$) for CONT than EXP; however, there were no differences ($P \geq 0.12$) in overall motility at other times. Progressive sperm motility tended to be reduced ($P = 0.07$) at week 2 and increased ($P = 0.08$) at week 7, but did not differ ($P \geq 0.15$) in other weeks. Serum testosterone concentrations were reduced ($P = 0.05$) by week 1 in EXP when compared to CONT; however, concentrations did not differ again until week 9 when EXP bulls had a 2-fold decrease ($P < 0.01$) in serum testosterone concentrations compared to CONT bulls. This steep decline at week 9 suggests a delayed effect of the pyrethroid and pyrethrin spray applications on peripheral testosterone concentrations. Further work, extending the project to include additional spermatogenic cycles, is necessary to evaluate the long-term effects of pyrethrin and pyrethroid insecticide applications on bull reproductive parameters.

Keywords: Pyrethroid, insecticide, bull, reproduction, fertility

Comparison of surgical vasectomy or chemical epididectomy as a sterilizing method for feral horse populations

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Introduction

Overpopulation of feral horses contributes to rangeland depletion and water source damage through fecal and urine contamination. They also suffer from starvation, dehydration and predation leading to welfare concerns. Sterilization is an important part of managing the feral horse population. While ovariectomy and immunization against porcine zona pellucida (pZP) have been used for mares, castration has been the only method used for stallions. This technique upsets the herd social hierarchy because dominant stallions are displaced. Surgical vasectomy or chemical epididectomy have been used in other species for male sterilization without altering social hierarchy. The objective of this study was to evaluate these methods for controlling feral horse populations. The hypothesis was that sterilizing stallions by surgical vasectomy or chemical epididectomy would be as effective as castration without reducing testosterone.

Methods

Stallions used in this study were part of the feral horse herd managed in accordance with the U.S. Fish and Wildlife Service comprehensive conservation plan for the Sheldon National Wildlife Refuge. Stallions were either surgically vasectomized (n=28), chemically epididectomized (n=16) or untreated (n=34) in the five years prior to this study. For the chemical epididectomy, 10 ml of 1% chlorhexidine in 90% DMSO (v/v) was injected into the tail of each epididymis. In 2013, stallions were gathered for routine castration. Prior to castration, venous blood samples were collected. Immediately following castration, a sample of fluid was collected from each vas deferens and evaluated for the presence of sperm by direct microscopic examination. Total serum testosterone concentrations were determined by chemiluminescence (Immulite ® 1000, Siemens). The effect of treatment on the presence of sperm in the vas deferens fluid was evaluated using Fisher's exact test and the effect of treatment on testosterone concentration (mean±SD) was evaluated using a Kruskal-Wallis rank sum test. Statistical tests were performed using R (version 3.0.2) and significance was defined as $p<0.05$.

Results

There was no significant difference in testosterone concentration between treatment groups (surgically vasectomized median 23.30 [25th and 75th percentile 20, 30.75] ng/mL; chemically epididectomized 29.25 [20, 58.325] ng/mL; untreated 20 [20, 20] ng/mL; Kruskal-Wallis $p=0.08$). Surgically vasectomized stallions had no sperm present in the remaining proximal vas deferens. All of the untreated stallions and chemically epididectomized stallions had sperm present within the vas deferens fluid samples. The difference in number of animals with the presence of sperm between treatment groups was highly significant ($p<0.001$).

Discussion

It is not clear why the chemical epididectomy was not an effective method for sterilizing the stallions. Additional research is needed on alternative methods (such as intratesticular injections) for non-surgical sterilization in feral horse populations.

Keywords: Epididectomy, feral horses, sterilization, testosterone, vasectomy

Can serum amyloid A predict reproductive efficiency in the postpartum of mares?

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Mares are often bred on the first estrus post-partum, termed foal heat (FH), and fertility is usually lower due to multiple factors such as poor uterine involution and chronic inflammation/infection. Serum amyloid A (SAA), an acute phase protein, has been shown to increase within 24 h of foaling followed by a rapid decrease to baseline concentrations by Day 3 postpartum. We hypothesized that concentrations of SAA would remain elevated beyond Day 3 postpartum in mares with uterine inflammation or infection. Moreover, we hypothesized that elevated SAA around the time of breeding would be indicative of impaired uterine involution, resulting in decreased fertility. Objectives were: 1) to determine if SAA concentration on Day 4 postpartum could be used to evaluate uterine involution; and 2) to determine if SAA concentration prior to breeding could predict fertility of mares bred shortly after foaling. Thoroughbred mares were bred on FH ($n = 8$) or on their second estrus postpartum (Control; $n = 13$). Mares with uncomplicated foalings occurring prior to April 22 were selected for breeding on their second estrus postpartum, while mares foaling at a later date were selected for FH breeding. Mares were monitored daily and bred once by natural cover while in estrus with good quality semen. All mares were evaluated on Day 4 postpartum and again on the day before breeding. Palpation and ultrasonography per rectum were used to assess uterine/cervical edema, tone, size and fluid accumulation. Endometrial cytology was evaluated and scored based on the average number of neutrophils per high-power field. Endometrial culture was evaluated after 48 h of aerobic incubation for growth of pathogenic bacteria. Blood was collected and serum was separated, frozen, and later evaluated for determination of SAA concentrations at the Acute Phase Laboratory at the University of Miami (Miami, FL). Data were analyzed using Fisher's Exact Test with significance set at $P < 0.05$. Overall, SAA concentrations were not correlated with the degree of uterine involution or fertility. On Day 4 postpartum, SAA was elevated (i.e. >50 mg/L) in 3/21 (14%) mares; however, only one mare (5%) had poor uterine involution. Moreover, 15/21 (72%) mares presented moderate to severe cytological evidence of uterine inflammation on the same day. On the day before breeding, SAA was below baseline in all mares in both groups. However, only 6/8 (75%) mares in the FH group had good uterine involution without cytological evidence of uterine inflammation compared to 13/13 (100%) of mares in the control group. Growth of pathogenic bacteria was observed in 16/21 (76%) mares on Day 4 postpartum. On the day before breeding, cultures from 2/8 (25%) FH mares yielded pathogenic bacteria compared to 0/13 mares in the control group. On Day 15 post-ovulation, 2/8 (25%) FH mares and 10/13 (77%) control mares were pregnant. Non-pregnant mares were bred on subsequent cycles and 6/9 became pregnant by the end of the season, indicating adequate fertility of mares in the study. Serum amyloid A was at baseline on Day 4 postpartum in 18/21 (86%) mares and did not correspond with pregnancy outcome. In conclusion, our results indicated that SAA was not a reliable indicator of uterine involution or predictor of fertility in mares.

Keywords: Serum amyloid A, mare, foal heat, fertility, horse

The effects of bovine sperm-bound anti-sperm antibodies on capacitation

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Sperm-bound anti-sperm antibodies (ASAs) can prevent sperm capacitation resulting in decreased fertility in men. However, their effect on bovine sperm capacitation is not known. The objective of this study was to evaluate the effect of sperm-bound ASAs on bovine sperm capacitation. It was hypothesized that ASAs negatively affect the ability of bull spermatozoa to undergo capacitation *in vitro*. Four yearling angus bulls with satisfactory breeding potential were used. First, three ejaculates (ASA negative) were collected and cryopreserved. Then, ASAs were induced by serial immunizations with autologous spermatozoa. Three more ejaculates (ASA positive) were cryopreserved starting three weeks after the last immunization. The percentage of IgG- and IgA-bound spermatozoa was assessed at the time of semen collection using flow cytometry. On the day of the experiment, straws from each ejaculate were thawed and divided into two aliquots. Sperm capacitation was induced in one aliquot by incubation in SP-TALP with heparin (60 µg/mL) at 37 °C in 5 % CO_2 in air for 45 min (HEP treatment). Another aliquot incubated without heparin served as the control treatment (CON). The percentage of capacitated live spermatozoa in each treatment group was determined via flow cytometry and the fluorescent stains merocyanine 540 and Yo-Pro1. The percentage of capacitated live spermatozoa was compared among ASA-negative CON, ASA-negative HEP, ASA-positive CON and ASA-positive HEP treatments using ANOVA for repeated measurements. Pearson's correlation was analyzed between the percentage of IgG- or IgA-bound spermatozoa and the percentage of capacitated spermatozoa after exposure to heparin. While the mean percentage of capacitated spermatozoa was higher in the HEP than CON treatments among ASA-negative samples ($P < 0.01$), there was no significant difference between HEP and CON treatments in ASA-positive samples (Table). The percentage of capacitated spermatozoa was negatively correlated with the percentage of IgG- ($P = 0.041$, $R^2 = -0.402$) and IgA-bound spermatozoa ($P = 0.02$, $R^2 = -0.429$).

Table. Sperm capacitation in ASA negative and positive ejaculates (Mean % ± SEM).

ASA group	Negative		Positive	
Treatment	CON	HEP	CON	HEP
Capacitated spermatozoa (%)	20.5 ± 2.3^a	48.1 ± 3.8^b	12.8 ± 4.5^a	31.8 ± 3.1^a

$^{a,b}P<0.01$;

In conclusion, sperm-bound ASAs impaired the ability of frozen-thawed bovine spermatozoa to undergo capacitation *in vitro*. While their effect on fertility needs to be tested, the inability of sperm to undergo capacitation may be a mechanism by which ASAs may contribute to bovine subfertility.

Keywords: Anti-sperm antibodies, capacitation, bovine, spermatozoa

Endometrial tissue concentrations of ceftiofur following intrauterine infusion in mares

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Evidence based guidelines on appropriate dosing intervals for intrauterine ceftiofur treatment of bacterial endometritis are lacking. The aim of this study was to determine endometrial concentrations of ceftiofur for 48 h following intrauterine infusion in a healthy uterus. It was hypothesized that endometrial tissue concentrations of desfuroylceftiofur-acetamide (DCA; active metabolite of ceftiofur) would remain above the MIC_{90} for *Streptococcus equi zooepidemicus (S. zooepidemicus)* and *Escherichia coli (E. coli)* for greater than 24 h.

Six clinically healthy, non-pregnant mares, of various breeds (age 5-21 years; weight 525-578 kg) were included in the study. Samples were taken during estrus for uterine biopsy, culture, and cytology. Only mares that were free from uterine pathology were included in the study (Kenney-Doig biopsy score of I or IIA, negative uterine cultures and less than 3 polymorphonuclear neutrophils per high power field on cytology). Endometrial biopsy samples were taken immediately prior to the intrauterine infusion of 1g ceftiofur (diluted in sterile saline to a total volume of 60 ml). Further endometrial biopsy samples were taken at 4, 8, 12, 24, 36 and 48 h after ceftiofur infusion. Tissue samples were stored at -20°C until analysis. Desfuroylceftiofur-acetamide levels in endometrial tissue were measured using liquid chromatography-mass spectrometry at each time point. Data were analyzed with a Wilcoxon signed-rank test.

At all time points, endometrial tissue concentrations of DAC were well above the MIC_{90} for *S. zooepidemicus* (0.25 mcg/ml)[1] and *E. coli* (0.5 mcg/ml)[1]. At 24 h DCA concentrations were different from the MIC_{90} for *S. zooepidemicus* and *E. coli*, (P =0.031, N=6, range 8-660 mcg/ml). At 48 h after infusion DCA endometrial tissue concentrations were still elevated above the MIC_{90} for *S. zooepidemicus* and *E. coli*, however one mare was removed from the study at 24 h (P =0.065, N=5, range 3-114mcg/ml).

The results of this study support the clinical use of once daily intrauterine infusions of ceftiofur. They also suggest that less frequent dosing may be appropriate as all mares tested at 48 h had levels above therapeutic concentrations. Further studies are required to determine the effects of inflammation on tissue concentrations of DCA and if endometrial tissue levels of DCA remain above therapeutic concentrations for greater than 48 h and therefore if even less frequent dosing intervals can be justified.

Keywords: Bacterial endometritis, ceftiofur, Endometrial biopsy

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Relative sperm volume shift, aquaporin 7 mRNA abundance and bull fertility estimates

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Integrity of sperm plasma membrane is important for sperm function. Mammalian sperm experience a natural osmotic decrease during male to female reproductive tract transition. This hypoosmotic exposure not only activates sperm motility, but also poses potential harm to sperm structure and function when improper swelling of spermatozoa occurs. Aquaporins (AQP) are a family of membrane channel proteins implicated in sperm osmoregulation. There are a variety of assays testing the membrane integrity of spermatozoa including the hypoosmotic swelling test (HOST). However, HOST has shown varied correlation with fertility. Alternatively, sperm volumetric parameters evaluate not only swollen cells, but also the degree of swelling. Given the varied correlation between HOST and fertility, our hypothesis is sperm volumetric measurement will better predict fertility than HOST and aquaporin7 mRNA abundance will putatively play a role in osmoregulation. The objective was to determine the association among relative sperm volume shift, % HOST, sperm AQP7 mRNA abundance and sire conception rate (SCR; fertility index) in Holstein bulls. The SCR estimate for the study bulls (N=34) was based on at least 500 services, (725±13 services/sire) and for each full point SCR score (from –4 to +4), 3 to 5 sires were included. Samples of frozen thawed sperm from a single collection from these commercial Holstein bulls were used to evaluate relative mRNA expression of AQP7 in sperm. Sperm cell volumetric measurements (using Image J1.42q and Quantity One 4.6 software) and % HOST (using microscope) were determined for 400 sperm after incubating the samples in isoosmotic (300 mOsm/kg) and hypoosmotic (100 mOsm/kg) solutions for 15, 30 or 60 min. The relative sperm volume shift ($Vr=V_{hypo}/V_{iso}$) was used as a measure of volume regulation in response to hypoosmotic conditions. Data were analyzed using SAS (Version 9.3 for Windows, SAS Institute, Cary, NC). Coefficient correlations were estimated using PROC CORR to determine the association of CT (threshold) values (dCT) for AQP7, % HOST and relative volume shift with individual bull SCR-scores. The RT-PCR data, $2^{-\Delta\Delta Ct}$ values for mRNA expression of AQP7, and relative sperm volume shift data were analyzed by ANOVA to ascertain statistical significances. There was no correlation between %HOST and SCR ($r=0.28$ $P>0.1$). There was a significant positive correlation between relative sperm volume shift and SCR ($r=0.43$ to 0.65, $P<0.05$). The AQP7 mRNA abundance was positively correlated to relative volume shift ($r=0.64$ to 0.73; $P<0.05$) and SCR ($r=0.67$; $P<0.05$). The mRNA expression of AQP7 and relative sperm volume shift differed among low (<2 SCR), average (-2 to +2) and high (>2) fertility sire groups ($P<0.05$). In conclusion, bulls with higher SCR had a greater quantity of AQP7 mRNA in frozen thawed sperm. This may have contributed to greater regulation of sperm volume shift which protected the sperm from detrimental swelling and impaired functions.

Keywords: Holstein bull, sire conception rate, sperm volume, HOST, aquaporin

Management of anejaculation in a stallion associated with neurologic deficits

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Anejaculation is the most common cause of ejaculatory dysfunction in the stallion.[1] A 9-year-old Andalusian stallion was presented with a history of normal libido, but refusal to either mount the phantom or inability to ejaculate when mounted on the phantom. The stallion had a two year history of hind limb weakness, intermittent urine dribbling, and inability to completely void his bladder. He tested negative for equine protozoal myeloencephalitis (EPM) in October 2011.

A lameness examination was normal, but a neurologic examination identified hind limb incoordination, including cross-cantering and 1.5/5 and 1/5 ataxia on the right and left pelvic limbs, respectively.

At presentation, the stallion did not ejaculate on the first two attempts and was weak when dismounting (knuckled over at the fetlock and slid off the phantom). Attempts at ground collection and chemically induced ejaculation were not successful. Four days after admission, the stallion did not urinate within a 24 hour period. Transrectal ultrasonography revealed a greatly distended bladder (>12cm depth). The bladder was catheterized and drained (12 L).

Multiple ejaculates were subsequently obtained using a combination of a live jump mare, a long Missouri AV and hot towels, concurrent with administration of phenylbutazone (BID). In addition, imipramine hydrochloride (1.0g PO) and gonadltropin releasing hormone (GnRH; 50µg IV) were given two hours prior to collection, with a similar dose of GnRH given one hour prior to collection.

Anejaculation is a relatively common condition in stallions and can be caused by neurologic deficits. This stallion exhibited neurologic limitations manifest by an inability to urinate, incoordination, and hind limb ataxia. Despite the deficits, accommodations to enhance sexual arousal such as imipramine,[2] GnRH,[3] and a live mare mount combined with enhanced penile stimulation (hot towels) were introduced that allowed semen to be collected routinely.

Keywords: Stallion, anejaculation, neurology

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Transrectal cranio-cervical dislocation of a twin fetus in a mare

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A 7-year-old Thoroughbred mare was diagnosed at 40 days gestation with twin fetuses adjacent to each other in the right uterine horn. At 66 days gestation, the mare was given flunixin meglumine (1 mg/kg IV), buscopan (0.4 mg/kg IV), altrenogest (0.044 mg/kg PO SID), and sedated with detomodine (0.006 mg/kg IV) and xylazine (0.3 mg/kg IV). Guided by transrectal ultrasound (7 mHz), the fetal head of the distal twin was stabilized transrectally between the thumb and forefinger. Dislocation was achieved by positioning the thumb at the base of the cranium and applying pressure side to side until a distinctive pop was felt and the thumb and forefinger could be placed between the head and the neck.[1] Evaluations were performed one and two days later showed active heartbeats in both fetuses. One fetus appeared normal but the damaged fetus had hyperechoic debris in the fetal fluid and the head was visualized detached from the body. No evidence of the reduced fetus was apparent on ultrasound at 151 days. At 333 days gestation, the surviving foal was born without incident. The fetal membranes showed no abnormalities and no sign of the reduced twin was noted. The foal showed obvious congenital angular limb deformities with the right metatarsus caudally displaced to the tarsus, valgus deformities of both front legs and left hind, and abnormal ossification, particularly of the tarsal bones. After corrective measures, the foal showed moderate improvement, but prognosis for a future as a show horse is guarded. The mare was rebred and confirmed to have a singleton pregnancy at 14 days gestation. Twin pregnancy is a major cause of pregnancy loss in horses. While early ultrasound diagnosis and transrectal manual reduction of the twin vesicle is very successful, reduction after Day 16 still provides a challenge to equine practitioners.[2-5]

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Hydroallantois and hydroamnios associated with a bulldog calf and abnormal placentation in a Miniature Scottish Highlander cow

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A 2 year-old pregnant Miniature Scottish Highlander cow was presented for suspected hydrops with an unknown breeding date. On presentation the cow had a large, distended abdomen. Transabdominal and transrectal ultrasound identified a large amount of hypoechoic fluid within the uterus and no viable fetus. Due to absence of fetal viability and a severely distended uterus, labor was induced.

A large amount of placental fluid was passed, but due to lack of progression, an ultrasonographic examination was repeated 48 hours after induction and abnormal hyperechoic placentomes and a non-viable fetus were visualized. A vaginal examination determined that the fetus was too large to pass vaginally and a cesarian section was performed. A dead bull calf with axial and appendicular skeletal chondrodysplasia , severe brachygnathism, and hydrocephalous, among other defects, was delivered. The abnormally formed fetus and lack of normal placentation could have both contributed to the accumulation of fluid that led to the presentation of hydrops amnion/allantois.

Chondrodysplasia is typical of the bulldog calf mutation that has been described in Dexter cattle. This defect has been associated with a gene mutation in the aggrecan gene ACAN. Both the dam and the sire were tested and found to be heterozygous carriers for this gene mutation. The clinical presentation, in combination with parental genetic testing, lead to the diagnosis of a bulldog calf mutation in this case. It was recommended to use a sire that tests negative for the mutation if this cow is to be re-bred.

This is the first reported case of the bulldog calf mutation in Miniature Scottish Highland cattle. This is of clinical importance because it demonstrates that the bulldog calf mutation is not limited to Dexter cattle, suggests that miniature breeds may be genetically similar and highlights the clinical importance of genetic testing for known diseases among cattle breeds.

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Segmental aplasia of the tubular reproductive tract in a Shorthorn heifer

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A 1.5-year old Shorthorn heifer was presented for evaluation of a persistent vaginal discharge and failure to conceive. The heifer had been serviced twice by a bull 35 days prior to presentation and developed intermittent vaginitis and vulvar discharge. She was treated with ceftiofur and estrus synchronization was attempted, but the controlled drug release (CIDR) insert could not pass completely into the cranial vagina. During presentation, all physical parameters were within normal limits except for presence of a yellow/white vulvar discharge. Palpation and ultrasonography per rectum revealed presence of a corpus luteum (CL) on the left ovary along with presence of anechogenic fluid within the uterine horns (i.e. mucometra) and fluid with echogenic particles within the uterine body (i.e. pyometra). There was no communication between the uterine horns and body. The cervix felt small and a cystic structure was visualized at the cranial aspect. On vaginoscopy the vagina appeared normal with no adhesions or bands. A structure resembling the external os of the cervix could be visualized and mucopurulent exudate was draining from it. Uterine catheterization was attempted but the catheter could not be advanced further than the external os of the cervix. The lack of communication between the uterine horns and body indicated that she had a segmental aplasia of the tubular tract, also known as White Heifer Disease. This condition is congenital and genetically inherited, as seen more often in Shorthorn breeds. In Shorthorns the disease is caused by a single, recessive, sex-linked gene linked to the gene for white color. Due to the heritability of this condition, the heifer should not be used in any reproductive capacity (e.g. oocyte donor). Although the prevalence of this condition is relatively low we, as theriogenologists, must be able to educate breeders in order to assure that undesirable traits are not passed on to offspring and perpetuated in the species.

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Breeding management in a reindeer (*Rangifer tarandus*) with a history of reproduction failure

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Artificial insemination is commonly used in domestic ruminants, but less commonly used in farmed cervids. Estrous synchronization timing and artificial insemination is particularly challenging in reindeer because there has been so little research in this area. This case report describes timed artificial insemination and two methods of pregnancy diagnosis in a reindeer with a history of reproductive failure.

In October 2012, an 8-yearold reindeer cow was artificially inseminated following estrous synchronization. Based on her body score and antler condition, she was in good general and nutritional health. In January 2013, she was diagnosed pregnant using a serologic test for pregnancy specific protein B (PSPB; BioPRYN®wild, BioTracking, LLC, Moscow, ID) with an optical density (OD) result of 0.3098 (pregnancy at OD > 0.21).[1] Pregnancy was reconfirmed in April 2013 by the same serologic test with an OD of 0.2554. However, she failed to calve at the expected due date in May and pregnancy loss was suspected just prior to the April PSPB serologic test since the OD was decreasing.[2]

In October 2013, the reindeer cow was administered a controlled internal drug release (CIDR) insert (Eazi-Breed ™ CIDR ®, Zoetis, Florham Park, NJ) and 50 µg of gonadotropin releasinghormone (GnRH; Cystorelin®, Merial LLC, Duluth, GA) intramuscularly. One week later, the CIDR was removed and 250 µg cloprostenol (Estrumate™, Schering-Plough Animal Health Corp., Summit, NJ) was administered intramuscularly. Forty-five hours later, frozen semen from a collection that had resulted in previous pregnancies was thawed and transcervically inseminated using a flexible endoscope. At the time of insemination, 50 µg GnRH was given intramuscularly. Artificial insemination was repeated ten hours later at the time of each insemination, estrous signs were observed.

At 6, 14, and 20 weeks after insemination, transrectal ultrasonography was performed to determine and reconfirm pregnancy. The cow is expected to calve in May 2014 (term gestation 225-235 days).[3]

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Unilateral uterine prolapse in a 7-year old multiparous cat

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An intact, 7-year old multiparous queen in a breeding colony was found with a uterine prolapse following the delivery of four live kittens overnight. The queen was systemically stable and the tissue was pink with moderate edema. An attempt was made to reduce the prolapse non-surgically under heavy sedation (dexmedetomidine) and spinal anesthesia (lidocaine), but failed. General anesthesia was induced and a ventral midline incision was made. The right uterine horn was located within the abdomen. The left horn was prolapsed out of the vagina through a tear in the uterine body. The horn was replaced back into the abdomen and an ovariohysterectomy was performed. Recovery was uneventful and the queen raised her kittens without incident.

Uterine prolapse usually occurs within 48 hours of parturition.[1] Whole uterine prolapse has been reported in cats from 10 months to 6 years in age[2] while unilateral prolapse is unusual in small animals. Uterine prolapse is considered an emergency with guarded prognosis if not addressed quickly due to hemorrhagic shock secondary to rupture of the ovarian or uterine vessels.[3,4]

Treatment options depend on the severity of prolapse, edema, and tissue damage. When minimal tissue damage is present, the uterus may be replaced vaginally either under general anesthesia or heavy sedation and epidural anesthesia. If there is significant edema or tissue necrosis, a ventral midline incision approach should be made allowing reduction of the uterus internally followed by ovariohysterectomy.[1]

Uterine prolapse does occur in the cat, but unilateral uterine prolapse is less common. This case demonstrates a positive outcome and survival following a unilateral uterine prolapse. Ovariohysterectomy was a successful treatment option for this cat due to the duration of prolapse and poor prognosis of future fertility based on the tissue condition and her age.

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Comparison between immunocastration and surgical castration in dogs

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Surgical castration is the traditional method of gonadectomy in male dogs and cats. However, welfare concerns have started growing regarding surgical castration. Administration of vaccines against gonaodotropin releasing hormone (GnRH) have been reported as an alternative to surgical castration in dogs and cats, although the side effects of this treatment are yet to be fully established. In this study we compared the effects of surgical castration and immunological sterilization, using criteria such as body mass, complications, testicular morphology, semen evaluation, complete blood counts (CBC), and plasma testosterone (T) and luteinizing hormone (LH) concentrations. Fifteen sexually active, intact male mongrel dogs were randomly divided into three groups of five dogs each. Group 1 (control) dogs were treated with an intramuscular injection of normal saline; dogs in group 2 (immuno-sterilized) were treated with a single injection of GnRH vaccine (Improvac, Pfizer Laboratories, South Africa) and dogs in group 3 were surgically castrated under ketamine-xylazine-diazepam anaesthesia. Semen was collected by manual stimulation, before the administration of the vaccine or surgical castration and thereafter weekly for eight weeks. Volume (SV), sperm concentration (SC), sperm motility (SM), live-dead ratio (LDR) and percentage of morphologically abnormal spermatozoa were determined for each ejaculate. Also, blood was obtained from the cephalic vein for the determination of CBC, T and LH. The dog's body weights (BW) were recorded weekly. Eight weeks after treatment the control and immuno-sterilized dogs were surgically castrated and their testes fixed in Bouin's fixative for histopathological examination. The BW, SV, SM, SC, T and LH were compared using analysis of variance (ANOVA) for repeated measures. The only adverse reaction noted in immuno-sterilized dogs was moderate swelling at the site of injection, while post-operative swelling and chewing at the incision site were observed in the surgically castrated dogs. While there was no significant ($p > 0.05$) difference in body weights between surgically or immuno-sterilized dogs, body weights of all treated dogs increased progressively until eight weeks after castration. There were no significant ($p > 0.05$) differences in PCV, WBC, T and LH between surgically castrated and immunized dogs. The mean values of SV, SC and SM ($P < 0.05$) decreased two weeks following treatment in immunized and surgically castrated dogs, and thereafter until dogs became azoospermic. The testes of control dogs were significantly longer than those of the immunized dogs (6.1 ± 0.7cm vs. 4.4 ± 0.3cm; $P < 0.05$). Histologically, the testes of the immunized dogs showed clear disruption in the number and morphology of the interstitial (Leydig) cells. At this stage it is widely assumed that immuno-sterilization in male dogs is reversible, implying that dogs must be regularly re-immunized to maintain their sterility. It was therefore concluded that immuno-sterilization does not have any advantage over traditional surgical castration except in areas where surgery is impractical.

Keywords: Castration, immuno-sterilization, testis, dogs

Comparison of penile spines and sperm morphology between juvenile and adult feral tom cats

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Background

Hundreds of thousands of dollars and volunteer hours are spent on trapping, neutering, and releasing feral cats with no apparent effect on the size of the population.[1] Therefore, we hypothesized that feral tom cats have reproductively adapted to man-made sterilization efforts by lowering the age at which they enter puberty and by increasing their fertility as compared to domestic cats. The objective of this study was to compare the presence of penile spines and the percentage of normal spermatozoa expressed from the vasa deferentes of juvenile and adult feral tom cats.

Methods

Tom cats were presented for castration at a feral cat neutering clinic. Age was estimated by a single observer. The age ranges were: 2-6 months (juvenile; n=13) and over 6 months (adult; n=16). After inducing general anesthesia, the penis was evaluated to determine if spines were present. Next, a routine open castration was performed. Contents from both vasa deferentes were milked onto a microscope slide, mixed with eosin-nigrosin stain, spread with a spreader slide, and allowed to air dry. The smears were blindly evaluated by the same observer (EB) using bright field microscopy under oil immersion (1000X) and the percentage of sperm with normal morphology was determined after evaluating 100 sperm per slide. The presence of penile spines was compared using a Chi-Square test and the percent morphologically normal sperm was compared using a Student's t test; where $p<0.05$ was defined as significant.

Results

More adult toms (16/16) than juvenile toms (4/13) had penile spines ($p<0.05$). For several toms (8/13 juveniles and 6/16 adults) fewer than 50 sperm were found on the slide. The results from these smears were therefore not included in the evaluation of sperm morphology. The mean ±SD percentage of morphologically normal sperm for the remaining five juvenile and ten adult tom cats was not significantly different (82±6% and 85±9%, respectively).

Discussion

Ives et al. described the eruption of penile spines to occur between 9 and 13 weeks of age.[2] It is not clear why more of the juvenile toms did not have penile spines present at the time of castration, but this may have been related to over-estimation of the age of the animals in our study.

Based on our findings we conclude that feral tom cats in this population do not reach puberty any earlier than toms in populations not subjected to population control measures, however we did find some evidence that the feral cats had better sperm morphology, one possible indicator of fertility, than has been reported for domestic cats (~70%)[3]. Further studies, comparing toms of known ages and both populations (domestic vs. feral) will have to be conducted to better test our hypothesis.

Keywords: Feline, Neuter, Puberty, Vas Deferens

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Evaluation of pseudopregnancy in the American black bear (*Ursus americanus*)

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The American black bear (*Ursus americanus*) exhibits several peculiar reproductive strategies that show high synchronicity with the environment. For instance, mating season occurs from June through August, months with the highest food productivity. Soon after, the fertilized embryos (blastocysts) arrest their development until mid-to-late November when implantation occurs. Thereafter, active gestation lasts 60 days. Delay in embryo development allows black bears to carry their pregnancy and give birth while hibernating over winter. Moreover, it is believed that black bears can experience pseudopregnancy. It is currently assumed that the primary corpus luteum is the main source of progesterone (P4) during active pregnancy until parturition. Previous studies conducted on various bear species, including black bears, have shown similar patterns in serum P4 profiles in females that gave birth and those that did not give birth. However, such studies in black bears did not include any pregnancy diagnoses, which could have resulted in false negative observations. Pregnant females that experience fetal death could display similar P4 profiles to those that produced live offspring. The aim of the study was to use serum P4 profiles to determine whether female bears that were never detected as pregnant by ultrasonography experience pseudopregnancy (defined as the maintenance of luteal P4 production, in the absence of pregnancy, for periods and concentrations similar those of pregnant females). Serum samples were collected from 8 female black bears (5 non-pregnant and 3 pregnant that did not give birth to cubs; mean ages 10.8 and 8yrs, respectively). Bears were captured by the Virginia Department of Game and Inland Fisheries and housed at Virginia Tech's Black Bear Research Center between October and February for various years from 1989 to 2005. During this time, bears were anesthetized every 10 days for ultrasonography and collection of blood samples. Blood samples were centrifuged and stored at -20°C until measurement of serum P4 by the ^{125}I Coat-A-Count progesterone immunoassay. Contrary to previous studies, P4 concentrations of pregnant and non-pregnant females were significantly different ($P<0.001$). During the pre-implantation period there was a 2 and 2.5 fold increase in P4 in non-pregnant and pregnant bears, respectively. During periods of active pregnancy concentrations of P4 were significantly different, with pregnant bears showing a 5-fold increase in P4 after embryonic implantation, while non-pregnant bears showed a gradual decrease in P4, returning to baseline within 20 days of the pre-implantation peaks. These results suggest that black bears are unlikely to exhibit pseudopregnancy.

Keywords: Bear, progesterone, pseudopregnancy, ultrasonography, implantation

Surgical correction of a perineal fistula in a 9 month old female Dandie Dinmont terrier with urinary incontinence

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A 9 month old female Dandie Dinmont Terrier presented to the Oregon State University Veterinary Teaching Hospital with a history of urinary incontinence. Urogenital examination revealed a 3 mm circular orifice in the perineum, midway between the vulva and the anus, through which urine leakage was observed. When examining the vulva, an excessively large clitoris was evident. The cause of the incontinence was assumed to be a developmental urogenital anomaly resulting from an intersex condition. Excretory urography by computed tomography (CT), followed by antegrade and retrograde CT urethrography was performed with a 64 slice helical CT scanner (Aquilion®, Toshiba Medical Software, Tustin, CA). Computed tomography showed a congenital caudal genito-urinary malformation with two urinary exit tracts that communicated between the urinary bladder and a fluid-filled uterus within the pelvic canal. The external urethral opening in the clitoris was smaller than the more dorsal perineal opening. The selected surgical approach was to resect the fistula, closing the extra perineal orifice. The clitoral urethral orifice was enlarged and the vulva was reconstructed to better envelop the clitoris (much like the prepuce covers the penis in the male animal). Postoperative perineal distension, erythema, and pain led to the decision to perform a radiographic positive contrast retrograde urethrogram, which revealed urine leakage and contrast pooling at the perineal surgical site. An ovariohysterocolpectomy and surgical repair at the site of urine leakage were performed during a second operation. The surgical repair revealed a failure in the closure of the urethral mucosa. Postoperatively, a 5 French red rubber catheter was placed in the urethra for 72 h to assist in the healing process by limiting hydrostatic pressure within the urinary tract. The previously observed perineal distension, erythema, and pain did not recur. The patient was discharged one week after the second operation and six months postoperatively was still continent and clinically normal. Histopathological evaluation of the surgically removed gonads revealed only ovarian tissue without evidence of a testicular component, thus ruling out hermaphrodism as a cause of the developmental disorder. Karyotyping confirmed a 78XX genotype. Further investigation also revealed that this animal was one of three females in a litter of five and provided no evidence of the dam's exposure to any reproductive steroids during gestation.

Urinary incontinence is a relatively common disorder, particularly in bitches. This case is notable because the urinary incontinence resulted from anatomic abnormalities of the internal and external urogenital structures without any apparent cause. The methods used to reconstruct the perineal area and correct the urinary incontinence in this case reflect an amalgam of those previously reported. In this case, the maintenance of the clitoral urethra (maximizing urethral length and theoretically increasing the likelihood of urinary continence), and the reconstruction of the vulva (serving as an envelope for the enlarged clitoris and preserving an environment for normal flora) represent novel aspects of surgical treatment.

Keywords: Urinary incontinence, clitoris, perineal fistula, intersex

Post-natal expression of cytochrome P450, family 26 genes in canine testis

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Cytochrome P450 (CYP) enzymes are a diverse group of catalysts that consist of several members in humans and animals. Cytochrome P450 26 class is involved in the metabolism of retinoic acid (RA), rendering this classical ligand inactive through oxidation. Spermatogenic or oogenic fate is dictated by the signals from the gonadal environment, in addition to the genetic mechanisms of sex determination. Gonadal environmental stimuli are primarily mediated by the selective exposure of RA ligand. Retinoic acid, therefore, plays a critical role in germ cell development. Cytochrome P450 26B1 degrades RA in the embryonic testis, preventing STRA8 expression, thereby delaying meiosis. Cytochrome P450 26B1 is thus required for the maintenance of the undifferentiated state of male germ cells during embryonic development, inducing arrest in the G0 phase of the cell cycle and preventing meiotic entry. Cytochrome P450 26B1 is considered a major catabolizing enzyme in embryonic and adult testes. However, CYP26A1, CYP26B1 and CYP26C1efficiently metabolize all-trans-RA to polar aqueous soluble metabolites. Therefore, we investigated the expression pattern of CYP26A1, CYP26B1 and CYP26C1 in canine testis. The objective of this study was to elucidate the gene expression of these enzymes in young, peripubertal and adult dog testes and to substantiate the gene expression pattern by protein localization of these enzymes in adult testis.

Gene expression patterns of CYP26A1, CYP26B1 and CYP26C1 were studied in young (N = 8), peripubertal (N = 6) and adult (N = 8) testes of mixed, medium-sized breeds using a real time polymerase chain amplification technique. SYBR green chemistry was employed, relative changes of gene expression in the young animal were calculated after normalization with the endogenous control, canine beta actin and the related fold changes were analyzed by ANOVA using 2 delta delta Ct values to ascertain statistical significance of any differences in gene expression. Protein localizations were examined using immunohistochemistry. These enzymes were tagged with primary antibodies on frozen adult testes sections. The ligand-primary antibody complex was then tagged with FITC-conjugated secondary antibodies. Images were captured using a white light laser confocal microscope.

Cytochrome P450 26B1 expressions were more abundant in young, peripubertal testes and adult testes compared to the mRNA expressions of CYP26A1 and CYP26C1. CYP26B1 expression was significantly lower at the peripubertal age. This finding implies that CYP26B1 is the major metabolizing enzyme in canine testis that controls the testicular RA level and spermatogenesis. Interestingly, lower expression of CYP26B1 at the peripubertal age supports its critical role in initiation of meiosis at puberty. Higher expressions at adult age suggest that other putative regulators also participate in maintaining the RA level in adult testis (in addition to CYP26B1) to ensure the continuous production of sperm. On immunohistochemistry images, CYP26B1 was mainly confined to the peritubular epithelial cells and interstitial area. Lower target signals were also observed for CYP26A1 and CYP26C1. The protein localization supports the functionality of the enzyme in the adult testis.

Keywords: Dog, testis, spermatogenesis, CYP26 family

Developing a non-surgical contraceptive method for female dogs

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An ideal contraceptive agent for dogs will induce permanent sterility and be effective in male and female animals of all ages. Previous studies have shown that nicotine has the potential to serve as a contraceptive agent. We therefore tested the hypothesis that nicotine will maintain the anestrous state in female dogs.

Two groups of four female beagle dogs, 1 year of age were studied. Placebo and nicotine pellets that release a total of 0.5 mg of nicotine per kg body weight per day for 90 days were implanted subcutaneously in the base of the neck, close to the shoulder. The treatments were repeated after 90 and 180 days. Vaginal cytology was performed every week to detect ovarian follicular activity. Blood samples were collected every week for 270 days to measure serum progesterone and estradiol concentrations. After a total of 270 days all animals were euthanized, and blood and tissue samples were collected.

Serum estrogen and progesterone concentrations were significantly reduced in nicotine treated females from two weeks after implant insertion until project completion (one way ANOVA: $p<0.05$). No significant difference in body weight was observed between the control and treated females. Daily physical examinations, temperature, pulse and respiratory rates, fecal and urinary analyses, feed intake and regular serum chemistry analyses revealed no adverse effects of treatment on the health of the dogs. Ovarian and uterine weights were lower in nicotine treated females. Histologic evaluation of ovarian sections revealed typical anestrous features in the ovaries of treated females while control animals were found to have estrous or diestrous ovaries.

These preliminary observations suggest that nicotine treatment has the potential to maintain female dogs in anestrus, without inducing significant side-effects. Further studies will be required to confirm that there are no adverse effects associated with long-term nicotine treatment.

Keywords: Nicotine, non-surgical, contraceptive, estrous, dogs

Comparison of canine spermatozoal RNA concentrations and purity using two density gradient centrifugation solutions

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Introduction

Sperm must be separated from other cells in the ejaculate prior to RNA isolation in order to provide a pure sample for analysis of genetic causes of infertility. Several methods for elimination of contaminating somatic cells have been described, including swim-up and density gradient centrifugation (DGC). In a previous study, we found the swim-up method yielded fewer morphologically normal sperm than DGC.[1] The objective of this study was to compare RNA concentration and purity following separation of dog sperm by DGC products commercialized for horses and cattle. Because of the two layer density gradient, we hypothesized that the cattle product would be more effective at removing somatic cells, yielding a purer sperm RNA sample.

Methods

Semen was manually collected up to six times from eight dogs and divided into three aliquots of equal volume. Two DGC products were used according to the manufacturer's instructions (Equipure™ and Bovipure™, Nidacon International, Mölndal, Sweden), while no cell separation technique was applied to the control samples. Total RNA was isolated using Trizol® reagent (Ambion®, Carlsbad, CA) according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically with a NanoPhotometer® (IMPLEN, Munich, Germany). Primers specific for canine sperm gene protamine-2 (PRM2) were designed using Primer3 software and information from the NCBI gene bank. Primers were synthesized by Sigma-Aldrich (St. Louis, MO). Gene specific transcripts were reverse-transcribed from the total RNA using Superscript® One-Step RT-PCR with Platinum® *Taq* kit (Invitrogen™, Carlsbad, CA) according to the manufacturer's instructions. Results were visualized on 2% agarose gels stained with SYBR® Green nucleic acid gel stain (Invitrogen™, Carlsbad, CA) utilizing the GelLogic 212 Pro Imaging System (Carestream Health, Woodbridge, CT). RNA concentrations and purity were compared between separation methods using a PROC MIXED platform in SAS (V.9.3, SAS Institute Inc, Cary, NC). Data were reported as least squares means±SEM.

Results

Sperm RNA concentrations did not differ between separation methods. However, both separation methods resulted in significantly greater amplification of the PRM2 transcript (purity) than the control. Results are summarized the Table. *$p<0.05$ compared to control

	Control	Equipure™	Bovipure™
RNA concentration (ng/µl)	60.50±19.30	94.76±25.73	78.90±19.22
Purity (100%=pure, 0%=contaminated)	23%±10%	60%±13%*	55%±10%*

Discussion

The two DGC products may have yielded similar results because the size the dog's sperm head (5µm x 7µm) is intermediate to that of stallions (3µm x 6µm) and bulls (5µm x 9.5µm).[2]

Keywords: Dog, ejaculate, primers, protamine-2, sperm separation

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Comparison of two electroejaculation methods in rams

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Introduction

A goal of the sheep producer is to select rams with the ability to impregnate most of the available females early in the breeding season. When collecting semen from a ram by electroejaculation, the dogma is that it is necessary to use a different stimulation technique than that described for bulls. The objective of this study was to evaluate two electroejaculation techniques for collecting semen samples in rams. Our hypothesis was that the "bull method" of electroejaculation used to collect semen from a ram would yield results similar to the traditional "ram method".

Methods

Suffolk and Dorset rams averaging 2 years old used in this study were part of a local flock. All were collected using a lubricated ram probe inserted into the ram's rectum with the electrodes facing ventrally and a Pulsator IV® electroejaculator (Lane Manufacturing. Denver, CO). Rams (n=48) were electroejaculated using either the "bull" method (n=24) or the "ram" method (n=24). For the "bull method", a pre-set program function was used that ran until a semen sample was collected. With the "ram method", downward pressure was also applied with the probe around the area corresponding to the accessory sex glands and massaged for three to four seconds during the rest period between stimulations. Then the probe was held stationary over the same area while a steady electrical stimulation was applied for seven seconds. This method was continued until a semen sample was collected. Samples were evaluated for motility and morphology using the guidelines set by the Society for Theriogenology. The Mann-Whitney test was used to evaluate the impact of these methods on response time and amplitude of stimulus required for the rams to ejaculate. The effect of method used on classification was evaluated using a Fisher's exact test. Statistical tests were performed using R (version 3.0.2) and significance was defined as $p<0.05$.

Results

There was significant difference in the time required for rams to ejaculate using the different methods. The "ram method" required an average of 45 (95% CI 15, 70) seconds longer to produce an ejaculate than with the "bull" method. There was no significant difference in the amplitude of electrical stimulation required between methods or between how the animal was classified (unsatisfactory or satisfactory). There were no "deferred" or "exceptional" classifications in either group of rams. Age and breed had no significant effect on classification, either alone or in combination with either method.

Discussion

Based on this particular group of rams, the "ram method" or "bull method" produced similar results with respect to efficacy of the method to produce a semen sample and quality of the samples collected when performing a breeding soundness examination.

Keywords: Ram, semen collection, breeding soundness examination, electroejaculation

Fatty acid composition of the yolk sac membrane in the chick embryo

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Background

Similar to the mammalian placenta with the chorioallantois and amnion, the avian yolk sac membrane (YSM) is comprised of the area vitellina (AVI) and area vasculosa (AVA), respectively. Understanding incorporation and delivery of nutrients (especially fatty acids) between these membranes and the developing embryo is important for improving hatchability and survival of chicks during early life. Due to its relative proximity to the yolk, we hypothesized that the AVI would be higher in fatty acid composition compared to the AVA.

Methods

Fertilized eggs from Ross broiler hens were incubated under standard conditions until embryonic day 16 (n=5) or day 19 (n=6) (hatching=day 20). To collect the YSM, the shell was cracked, albumen separated out, and the embryo within the YSM was placed into a sterile dish. The chick was then euthanized by cervical dislocation. The AVA was separated from the AVI and washed with phosphate buffered saline (PBS). The AVI was incised to facilitate removal of the yolk and washed with PBS. Membranes were separately flash frozen in liquid nitrogen. Total lipids were extracted from the AVI and AVA by the method of Folch et al.[1] Fatty acid methyl esters were prepared as reported by Metcalfe et al.[2] Fatty acid analysis was performed using gas chromatography as described by Cherian et al.[3] Total (mean ± SD) saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) were compared between the different membranes at each time point using one-tailed t tests. Significance was defined as $P<0.05$.

Results

Results are summarized in the table below. On day 16, the AVI had higher SFA and PUFA, but lower MUFA when compared to the AVA (*$p<0.05$). There were no differences found between fatty acid composition in AVA and AVI on day 19.

	AVI day 16	AVA day 16	AVI day 19	AVA day 19
SFA	41.2 ± 1.3*	39.2 ± 0.7	39.6 ± 1.3	39.3 ± 1.0
MUFA	34.5 ± 1.6	39.4 ± 1.3*	36.6 ± 4.3	40.1 ± 2.4
PUFA	24.3 ± 0.9*	21.4 ± 1.1	23.8 ± 3.7	20.6 ± 1.8

Discussion

These data suggest that the AVI is the primary membrane for providing essential PUFA for cellular signaling and SFA to meet the high energy requirements for embryogenesis. However, the role of MUFA in the AVA on embryo development requires further investigation.

Keywords: Area vasculosa, area vitellina, embryogenesis, hatchability

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Cystic endometrial hyperplasia with hydrometra and inappropriate lactation in a Nigerian dwarf goat, a case report

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Cystic endometrial hyperplasia (CEH) is caused by an increase in number and size of endometrial glands due to progesterone (P4) stimulation of the uterus at an inappropriate time in the reproductive cycle. This condition might result in fluid secretion by the glands into the uterine lumen, causing hydrometra, mucometra, or pyometra. This pathology has been well described in dogs, but is rare in other domestic animals. In dogs, CEH is the result of P4 acting on the endometrium in diestrus, after stimulation by estrogen during the proestrus period. This causes proliferation of the endometrium while the cervix remains closed under the influence of P4. In small ruminants, it is possible that prolonged estrogenic stimulation is involved similar to the pathology in the bitch. In the doe, elevated estrogen levels may result from follicular cysts, granulosa cell tumors, pituitary adenomas, or ingestion of estrogen-containing plants. In this case, an 8-year-old Nigerian dwarf goat doe was presented to the University of Georgia Veterinary Teaching Hospital for a two-week duration of pelvic limb paresis, and abnormal mammary gland development. Physical examination revealed a fully distended udder with milk expressible from both teats. The doe was diagnosed with *Parelaphostrongylus tenuis,* by analysis of cerebrospinal fluid, and treatment with flunixin meglumine and fenbendazole was initiated. The *P. tenuis* infection was the likely cause of the neurological signs; however, it did not explain the inappropriate lactation. Transabdominal ultrasound examination revealed multiple loops of fluid-distended uterine horn, and an increased area of echogenicity on the left ovary, consistent with luteal tissue. Based upon the ultrasonographic findings, pseudopregnancy and hydrometra (cloud burst) was the primary differential for the inappropriate lactation. The doe was administered two doses of 5 mg of dinoprost, three days apart in an attempt to produce a sustained luteolytic effect. A small amount of vaginal discharge was observed approximately 24 hours after the first injection; however, there was no reduction in the uterine size over the next 48 hours, evidenced by ultrasonography. The goat was humanely euthanized using a sodium pentobarbital injection due to worsening of neurologic disease, and was subsequently necropsied. Gross reproductive findings included severe distension of the uterine horns with a slightly cloudy uterine fluid containing scattered small caseous precipitate. Histologically the endometrium was hypercellular with luminal papillary projections, and contained cystic structures lined with a single layer of tall columnar epithelium. Scattered collections of eosinophils and dilated glands containing neutrophils and bacteria were seen throughout the lamina propria. The ovaries contained a follicular cyst and multiple corpora lutea (CL). It is hypothesized that this pathology might be the result of CEH due to continuous estrogen stimulation from a follicular cyst. Moreover, lack of a luteolytic signal (possibly influenced by endometrial pathology), could cause persistence of the CL and prolongation of P4 stimulation to the endometrium, which might also be a contributing factor for CEH in this doe.[1] Cystic endometrial hyperplasia may have subsequently predisposed this doe to hydrometra and pseudopregnancy, leading to inappropriate mammary development and lactation. Hydrometra is not commonly combined with CEH. Based on these findings CEH should be considered as a differential diagnosis in does with hydrometra.

Keywords: Cystic endometrial hyperplasia, pseudopregnancy, hydrometra, inappropriate lactation.

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Comparison of two superovulation protocols in the dromedary camel (*Camelus dromedarius*)

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Multiple ovulation and embryo transfer (MOET) has increased in practice, particularly in racing camels. Ovarian superstimulation methods remain poorly studied. Response the superovulation remains variable. We hypothesized that initiation of treatment at a specific day after ovulation would result in improved results than the traditional treatment. The objectives of this study were to compare ovarian response and embryo yield in donor camels superstimulated with follicle stimulating hormone (pFSH) following either progesterone treatment or at a specific time after ovulation.

Twenty multiparous healthy females were assigned to two treatment groups. Females in both groups received the same pFSH (Folltropin®, 20 mg/mL, IM, NIH-FSH-P, Bioniche, Pullman, WA) protocol: twice daily (am/pm) in decreasing doses (60/60 mg, 50/50 mg, 40/40 mg, 30/30 mg, 20/20 mg). Group 1 (n=10) received pFSH treatment starting 3 days after induction of ovulation with 100 µg gonadotropin releasing hormone (GnRH). Gonadotropin releasing hormone was administered when a mature follicle (12 to 18 mm in diameter) was present and there was increased uterine tone and edema. Group 2 (n=10) received pFSH after a 14-day course of progesterone in oil (100 mg/day, IM). Additionally, females in Group 2 received two doses of 500 mcg cloprostenol at 12 hour intervals on day 7 after induction of ovulation to ensure luteolysis. Ovarian follicular activity was monitored every other day by transrectal ultrasonography with a 7.5 MHz linear-array transducer during treatment and females were mated once to one of three proven fertility bulls when the diameter of the follicles reached 12 to 14 mm. All females received GnRH (100 µg, IM) and human chorionic gonadotropin (1500 IU, IV) immediately after mating. Presence of corpora lutea was detected by transrectal ultrasonography on day 8 after mating (day 0 = day of mating) and the uterus was flushed for embryo recovery using a non-surgical technique.

There was no significant difference between the two treatments in the interval from the last pFSH treatment to mating (7.5±0.5 days for Group 1 vs. 7.5±1.2 days for Group 2) and number of follicles at the time of mating (15.7±6.2 vs. 10.4±5.9). The number of corpora lutea at the time of embryo collection (12.8±4.3 vs. 8.2±4.3) and the number of transferrable hatched blastocysts (6.9± 3.0 vs. 3.8± 2.7) were significantly higher ($P<0.05$) in Group 1.

This study demonstrated that initiation of pFSH treatment three days after induction of ovulation may be more efficient for MOET in camels than initiation of treatment after 14 days of progesterone administration. However, embryo yield for both treatments remains highly variable. Further research is needed to develop a more predictable control of the follicular wave and superstimulation response in camels.

Keywords: Embryos, camelids, follicular stimulation, synchronization

Evaluation of a Monday-Friday 4-day CIDR + timed artificial insemination protocol in dairy heifers: a pilot study

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In dairy heifers, the application of a 5-day Co-Synch+controlled internal drug release (CIDR) protocol and timed artificial insemination (TAI) has resulted in acceptable pregnancy per TAI (P/TAI) ≥ 52.2 %;).[1] Recent studies showed that the initial gonadotropin releasing hormone (GnRH) at CIDR insertion and the second prostaglandin F2α (PGF2α) 12 h after CIDR removal are not essential to optimize P/TAI in dairy heifers subjected to a 5-day CIDR TAI protocol.[2] A major factor limiting the establishment and continuity of these programs by dairy producers is to overlook the significance of applying the hormonal injections at the correct day and time according to the specific protocol. This situation becomes more critical when trying to avoid cattle management practices during weekends. The establishment of a 4-day CIDR+TAI protocol performed on a Monday-Friday schedule would simplify the routine reproductive management of heifers in dairy farms, since it would facilitate treatment administration (Monday: CIDR insertion; Friday: CIDR withdrawal+PGF2α; next Monday: GnRH+TAI). We hypothesize that shortening the length of a CIDR-based TAI synchronization protocol by one day (Monday-Friday 4-day CIDR+TAI) will result in adequate P/TAI similar to that of 5-day CIDR+TAI protocols in dairy heifers. The objective of this study was to evaluate the P/TAI in dairy heifers treated with a Monday-Friday 4-day CIDR+TAI protocol compared to that of heifers treated with a 5-day CIDR+TAI protocol or AI after PGF2α injection and heat detection. A total of 45 Holstein heifers, 12-14 mo of age were randomly assigned to one of the three treatment groups: 1) Monday-Friday 4-day CIDR+TAI (Short-Synch, n=15): Heifers received an intravaginal CIDR insert (Eazi-Breed CIDR®, Zoetis Animal Health, Florham Park, NJ) containing 1.38 g of progesterone for 4 days. At the day of CIDR removal 25 mg of PGF2α (Lutalyse ®, Zoetis Animal Health) was injected intramuscularly (IM); 72 h after CIDR removal, heifers received 100 µg of GnRH (Cystorelin®, Merial Animal Health, Duluth, GA) IM and TAI. 2) 5-day CIDR+TAI (n=15): Heifers received the intravaginal CIDR insert for 5 days. On day 5, CIDR was removed and 25 mg of PGF2α was given IM; 72 h later, heifers received 100 µg of GnRH IM and TAI. 3) PGF2α-HD (n=15): Heifers were administered 25 mg of PGF2α IM and AI was performed 12 h after heat detection. Heifers were AI by an experienced technician, using conventional frozen-thawed semen from one of three sires (evenly distributed among groups). Animals were on pasture, with access to portable shades and trees, and fed a ration once daily that meets or exceeds the nutritional requirements of Holstein heifers. Pregnancy diagnosis was performed by transrectal ultrasound 36 days after AI. Data were analyzed using proc logistic and χ^2 test of the Statistical Analysis System (SAS®). Heifers in the 4-day CIDR+TAI group showed an optimal P/TAI (66.7%, 10/15) which was not significantly different from that observed in the 5-day CIDR+TAI (46.7%, 7/15) and PGF2α-HD (46.7%, 7/15) groups. In conclusion, the Monday-Friday 4-day CIDR+TAI 'Short-Synch' protocol resulted in adequate P/TAI in dairy heifers, similar to that of 5-day CIDR+TAI protocol or AI after PGF2α injection and heat detection. This protocol might represent a promising hormonal treatment for TAI in dairy heifers, facilitating their reproductive management routine, while maintaining an optimal fertility.

Keywords: 4-day CIDR, heifers, pregnancy, timed artificial insemination.

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Effect of 14 d CIDR treatment prior to 7 d CIDR CO-Synch

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The major goals of all timed artificial insemination (TAI) programs used in beef cattle are to achieve high conception rates and maintain a relatively concise calving season. This can only be accomplished when the cows are cycling. There are numerous factors that affect cyclicity of the beef cow: days postpartum, age, body condition, sucking reflex, presence of the bull, and the use of exogenous hormones. Various pre-synchronization protocols involving gonadotropin releasing hormone (GnRH), prostaglandin F2α (PGF) and controlled internal drug release (CIDR) have been applied to dairy cows to improve their cycling activity, but less has been performed in the post-partum beef cow. The objective of the current study was to apply a pre-synchronization protocol involving placement of a 14 d CIDR 26 d prior to the start of a 7 d CIDR CO-Synch to determine if this would improve TAI pregnancy rate (AIPR) as well as overall season pregnancy rate (SPR). Commercial beef cows (n=1770) across 15 locations (herd size ranges from 49-287; seven fall-calving herds, eight spring-calving herds) were used in the study. To be included, cows must have been seven days post-partum at CIDR 1 insertion (n=1555). Cows were randomly assigned to Treatment (14 day CIDR; TG) or Control (no CIDR; CG) groups and balanced for age and days post-partum (DPP). Body condition score (BCS) was recorded at CIDR 1 insertion. All cows were artificially inseminated (AI) with one of 12 sires (each sire was not used in every herd) using a 7d CIDR CO-Synch protocol. Herd bulls were turned out three days after AI and removed after 60 d. Pregnancy diagnosis via transrectal palpation/ultrasonography was performed twice at 40-60 and 100-120 days after AI, respectively. The data were analyzed using PROC GLIMMIX in SAS (Cary, NC). For AI pregnancy rate, unit (season), AI sire, age, and treatment (T) were included in the model. In the analysis for season pregnancy rate, AI sire was not included in the model. Additionally, BCS and DPP were covariates. The following interactions were analyzed: T by Age, T by DPP, and T by BCS. Significant differences were noted at $p<0.05$. For AICR, there was no significant difference between T (61.2%; n=781) and C (60.2%; n=773). Overall AICR of 2 yr. olds was notably ($p<0.05$) lower (58.2%; n=280) compared to 3 yr old (67.9%; n=224) and 4+ yr. old (60.3%; n=1045) cows. Also, the AICR was higher for increased DPP: <60 d (52.7%), 60-90 d (62%), and >90 d (60.6%). Unit, BCS, and treatment had a significant effect on SPR. Cows with a BCS greater than 5 (BCS 5-6 and 7-9) had higher SPR (93.7%; n=1087 and 87.4%; n=334, respectively) than BCS 2-4 (84.5%; n=116). The TG group had a higher ($p<0.05$) SPR (93.5%; n=781) compared to the CG group (89.8%; n=773). There are numerous conclusions that can be described from this study, but the overall premise is that a 14 day CIDR inserted 26 days prior to synchronization and TAI did not have an effect on AIPR but had a significant effect on overall SPR. This protocol appears to be beneficial for improving AIPR of young cows as well as overall SPR in these herds.

Keywords: CIDR, CO-Synch, artificial insemination, pregnancy rate

Evaluation of vaginoscopy in ewes utilizing the SILS™ port

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Introduction

Laparoscopic single site surgery is a technique where laparoscopic surgery is performed exclusively through a single entry point. The SILS™ port (Covidien, Mansfield, MA) has been previously described in the veterinary literature. This malleable port can accommodate three 5 mm or two 5 mm cannulas and one 12 or 15 mm cannula along with a dedicated port for insufflation. Our purpose was to evaluate the technical feasibility and reproducibility of transvaginal access and vaginoscopy using the SILS™ port device within ewes. We hypothesized that the SILS™ port would be a simple and safe alternative to traditional vaginoscopy approaches and provide appropriate visualization of vaginal structures, aiding in vaginal procedures such as biopsies.

Materials and methods

Eight healthy, mature ewes (four to eight years old; n=8) were used in this study. Animals were placed under general anesthesia in dorsal recumbency. The vulvar region was clipped and prepared prior to vaginoscopy. The pliable SILS™ port was manipulated through the vulva using a hemostat until securely in place extending from the vulva to the vaginal fornix. Three cannulas, two 5 mm (instrument cannula) and one 12 mm cannula (endoscope and camera), were inserted through the holes of the SILS™ port (Figure). Carbon dioxide insufflation was maintained at approximately 9 mm Hg. The vaginal vault was explored and vaginal biopsies were performed. Digital video and still images were recorded in each animal.

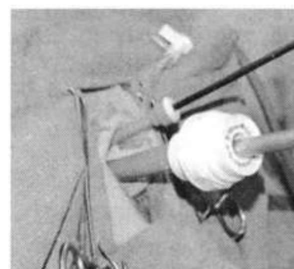

Figure. SILS™ port in vulva

Results

Placement of the SILS™ port was technically easy to achieve. The SILS™ port provided consistent visualization of the entire vaginal vault, including the cervix, in all eight ewes. The SILS™ port provided an effective seal to allow insufflation of the vaginal vault, which was required for complete visualization. The two 5 mm instrument cannulas were successfully utilized for mucosal biopsies in all eight ewes. Varying the insufflation pressures aided in the manipulation of vaginal tissues. No adverse events occurred following vaginoscopy using the SILS™ port in all ewes.

Discussion

This report demonstrates that the SILS™ port is a feasible, safe, simple and intuitive technique for performing vaginoscopy in ewes. The use of the SILS™ port provides a tight seal for insufflation, which is vital for consistent visualization of the entire vaginal vault. It also allows for the passage of two instruments into the vaginal vault for diagnostic or therapeutic procedures. While a rigid endoscope was utilized in this study, the SILS™ port can accommodate a flexible endoscope of equivalent diameter. Quality video and still images can be easily obtained using this technique for teaching or client education purposes. Further evaluation of the SILS™ port is warranted in other animals as a feasible vaginoscopy technique.

Keywords: Vaginoscopy, SILS™ port, endoscopy, single-port laparoscopy, vaginal biopsy

Comparison of two semen extenders for cryopreservation of Bighorn sheep (*Ovis canadensis canadensis*) epididymal sperm

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There is a growing amount of interest in the ability to preserve genetics of wildlife and endangered species, such as bighorn sheep (BH). Cauda epididymal sperm preservation is commonly used to salvage genetics from dead or terminally sick males. We have used the technique successfully with a commercial ovine extender[1] but this product is no longer available. The objective of this study was to compare the post-thaw quality of BH epididymal sperm using two semen extenders. Post-thaw evaluation included progressive motility, membrane integrity (using the hypoosmotic swelling test (HOST)) and acrosome integrity (using Spermac® stain).

Five bighorn rams aged 1-3 years were castrated using a closed technique. Anesthesia was achieved with an IM injection of Telazol® 5 mL (50 mg tiletamine and 50 mg zolazepam, Zoetis, Flrham Park, NJ) reconstituted with 250 mg (2.5 mL) xylazine and 2.5 mL sterile water and dosed at 4.4 mg/kg Telazol® and 2.2 mg/kg xylazine. The duration of anesthesia was approximately 30 minutes after which tolazoline was administered as a reversal agent. Epididymides were dissected from the testes within three hours of castration, and spermatozoa collected by the float-up technique.[1] Left and right epididymides were randomly assigned to be frozen in either the commercial bovine extender Triladyl® (Minitube of America, Verona, WI) or INRA96® (IMV Technology, St Paul, MN) with added 20% egg yolk and 5% glycerol. Two yearling rams were removed from the study because of poor initial semen quality. Recovered sperm was further diluted to a concentration of 200 million/mL and frozen as previously described.[1] Straws were thawed at 37°C for 30 seconds prior to evaluation in triplicate. Results of post-thaw quality are shown in the table below.

Table. Effect of extender on post-thaw parameters (Mean ± SEM) of BH epididymal sperm quality. Different letters in rows indicates a significant difference ($p<0.05$).

Post-thaw Parameters	Triladyl®	INRA96®+ EY + glycerol
% Progressive motility	65.6 ± 1.5^a	54.4 ± 3.7^b
% Intact membranes (HOST)	75.6 ± 3.1^a	58.3 ± 2.6^b
% Acrosome integrity (Spermac®)	72.9 ± 3.8^a	55.0 ± 2.6^b

In conclusion, post-thaw quality of BH epididymal sperm is higher using Triladyl®. However, INRA96 + 20% egg yolk + 5% glycerol provided acceptable post-thaw quality and could be used as an alternative. Further research on the fertility of frozen-thawed BH ram cauda epididymal sperm using these extenders is warranted.

Keywords: Epididymal sperm, cryopreservation, extender, castration, anesthesia

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Comparison of Serum amyloid A concentrations in Thoroughbred mares experiencing embryonic loss and in mares maintaining pregnancy

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Serum amyloid A (SAA) is a sensitive marker for acute inflammation and is increased in mares with experimental placentitis and bacterial endometritis. Elevations in SAA have also been reported in mares with early embryonic loss.[1] The objective of this study was to determine SAA concentrations in mares undergoing embryonic loss (< 42 days) compared to herdmates that maintained pregnancy. Serum amyloid A concentrations were determined at ovulation (Day 0) and at Day 15 postovulation in Thoroughbred mares in central Kentucky. Mares (n = 49) with confirmed embryonic loss were matched by age, reproductive status, farm, and veterinarian to mares that maintained pregnancy beyond 42 days (n=61). Serum amyloid A concentration was determined with a previously validated immunoturbidometric assay. Data were analyzed with a Wilcoxon rank sum test. There were no differences ($P > 0.05$) in SAA concentrations at Day 0 or Day 15 between mares that maintained pregnancy and mares that underwent embryonic loss. Median (interquartile range) concentrations of SAA at Day 0 were 31.9 mg/L (27.6 - 36.5 mg/L) for mares that maintained pregnancy versus 32.0 mg/L (29.8-37.0 mg/L) for mares that underwent embryonic loss. Similarly, median concentrations of SAA at Day 15 were 30.3 mg/L (27.4 - 32.8 mg/L) for mares that maintained pregnancy versus 29.4 mg/L (27.1-33.6 mg/L) for mares that underwent embryonic loss. Elevations in SAA were observed in mares from both groups but did not appear to be associated with embryonic loss. Based on our findings it appears that in this population of mares, pregnancy loss prior to 42 days was not associated with an elevation in SAA levels at the time of ovulation and first pregnancy diagnosis.

Keywords: Equine, mare, serum amyloid A, embryonic loss, endometritis

Acknowledgments

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Necrosis of the spermatic cord, epididymis, and testis of a Friesian stallion caused by *Salmonella abortus equi*

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Importance

Trauma, inguinal herniation, and torsion of the spermatic cord are causes of inflammation of the spermatic cord, epididymis and testis of stallions. Epididymo-orchitis characterized by coagulative and liquefactive caused by *Salmonella* spp. has been reported to occur in humans, rams, and chickens, but we can find no similar reports in the stallion. This case report describes a stallion affected with epididymo-orchitis caused by *Salmonella abortus equi.*

Diagnostic approach and treatment

A 5-year-old Friesian stallion was presented to the University of Tennessee's Veterinary Medical Center because of discomfort and enlargement of the right testis first observed four days previously. Examination revealed a thick plaque of edema and an enlarged right testis and spermatic cord. Palpation of the testis did not elicit signs of pain from the horse. Ultrasonographic examination, using Doppler, revealed hyperechoic areas in the right cord and testis consistent with necrosis. The right testis was moderately enlarged (7.1 cm diameter), and fluid was observed between the visceral and parietal tunics. The distal 3 cm of the spermatic cord had no blood flow. Testicular necrosis caused by torsion of the spermatic cord was suspected, and unilateral orchidectomy was recommended.

Results and discussion

During intra-operative examination, the right spermatic cord was observed to be oriented properly, but its distal 3 cm was necrotic. The cord and testis were edematous and contained areas of fibrosis. Scrotal fascia was thickened, and the parietal tunic was 6 to 7 mm thick and adhered to the testis. The testis and necrotic portion of the spermatic cord were excised using a closed technique of orchidectomy. The scrotal incision was sutured. Culture of foul-smelling exudate contained within the testis produced >500 colonies of *Salmonella abortus equi.* Severe, chronic, diffuse, necrosuppurative orchitis and epididymitis, and bacterial rods were observed during histological examination of the testis. The scrotal incision healed by first intention. The stallion returned to breeding after two months of sexual rest. Bacterial culture of semen collected from the stallion on three occasions during the next three months produced *Salmonella abortus equi.* Mares artificially inseminated with this semen became pregnant.

Keywords: Stallion, epididymitis, testis, orchitis, salmonella

Follicular dynamics following cloprostenol administration in the diestrous mare

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Prostaglandin F2α (PGF) treatment is routinely used in the reproductive management of mares to induce luteolysis and a subsequent return to estrus. The objectives of this study were to 1) evaluate the interval from administration of a single, 250 μg intramuscular dose of cloprostenol sodium (Estrumate® Schering-Plough Animal Health Corp., Summit, NJ) to ovulation in the mare with considerations of follicle size, mare age, and season at time of administration; 2) evaluate the fate of large follicle (≥35 mm) at the time of PGF administration; and 3) determine incidence of hemorrhagic anovulatory follicle (HAF) formation following PGF administration in mid-diestrus to 275 American Quarter Horse mares. A total of 529 estrous cycles were evaluated. Continuous data were compared using a one way ANOVA (analysis of variance) with post hoc analysis by student's *t*-test. Significance was set at $p < 0.05$. Categories were analyzed by Chi Square analysis. Data are presented as mean ± S.E.M. The average interval to ovulation following cloprostenol administration was 8.4 ± 2.5 days. The interval from PGF administration to subsequent spontaneous ovulation was inversely proportional to the diameter of the largest follicle at the time of treatment, if the follicle went on to ovulate (Table). In some mares, the largest diestrous follicle regressed and was replaced by another follicle on either the ipsilateral or contralateral ovary. Large diestrous follicles (≥35 mm) had one of three outcomes following PGF treatment: ovulation within 48 hours in the absence of uterine edema (14.5%), ovulation after 48 hours accompanied by uterine edema (75.4%), or regression without ovulation followed by emergence of a new follicular wave (10.1%). Hemorrhagic anovulatory follicle formation occurred in 12/529 cycles (2.3%) following PGF administration. No significant differences in mare age or season on outcome following PGF administration were noted. In summary, assessment of follicle size at time of PGF administration can be used to estimate the interval to subsequent ovulation. The incidence of HAF formation after PGF administration was low compared to results of a recently published report.[1]

Table. Interval to subsequent ovulation based on follicle size at time of PGF administration.

Follicle Size (mm)	Number of Cycles	Average day to ovulation
10 to 14	113	9.6 ± 0.2^a
15 to 19	84	8.6 ± 0.2^b
20 to 24	88	7.6 ± 0.2^c
25 to 29	30	6.7 ± 0.4^d
30 to 34	15	4.9 ± 0.4^e

[a,b,c,d,e] Data within column with different superscripts are significantly different ($p<0.05$)

Keywords: Prostaglandin, cloprostenol, luteolysis, mare

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Effect of early medium pH on equine embryo development in vitro after intracytoplasmic sperm injection

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Intracytoplasmic sperm injection (ICSI) and in vitro embryo culture are used to produce equine embryos for both clinical and research use, but little information is available on factors affecting embryo development in this system. In vivo, stallion sperm are exposed to a high pH (7.7 to 8.0) in the uterine fluid,[1] and in most species oviductal pH equals or exceeds that of the uterus. Accordingly, marked sperm protein tyrosine phosphorylation occurs only when the environmental pH approaches or exceeds 8, suggesting that this may be the physiological pH of the equine oviduct.[1] If so, the new zygote is likely also exposed to a high pH in the period immediately following fertilization. We hypothesized that exposure to high pH (~ 8) during sperm preparation and the first 2 h incubation of zygotes after ICSI may be beneficial to fertilization and cleavage rates. For this study, to maximize the ability to demonstrate an effect of treatment on early embryo development, we utilized sperm from a stallion shown to have suboptimal cleavage rates after ICSI under standard ICSI conditions (63%, vs. 95% for control stallion).[2] Oocytes were obtained by ultrasound-guided transvaginal follicle aspiration from live mares. In vitro-matured oocytes were injected with sperm via Piezo drill, then presumptive zygotes were cultured in human embryo culture medium (LifeGlobal; GB) containing 10% FBS; 20 mM glucose was added at Day 5. Embryos were examined for cleavage on Day 5 and for blastocyst development on Days 7 to 11. A high-pH and a standard-pH treatment were used. To achieve this, sperm preparation (swim-up) and the first 2 h of embryo culture were performed in bicarbonate-containing media (modified CZB and GB, respectively) incubated either in air (high-pH treatment) or in 5% CO_2 in air (standard-pH treatment). Media were equilibrated in their respective incubator for a minimum of 2 h before use. The pH values of incubated media were 8.0-8.4 (air) vs. 7.4-7.5 (5% CO_2 in air). Following the 2-h culture period, all embryos were placed in standard conditions (6% CO_2, 5% O_2 and 89% N_2) for the remainder of culture. There was no significant difference in cleavage rates between the high-pH and standard-pH treatments (22/30, 73% and 19/25, 76%, respectively). The blastocyst rate per injected oocyte in the high-pH treatment was significantly lower than that in the standard-pH treatment (0 vs. 24%, respectively; $P < 0.01$). High pH (8 to 8.4) during sperm swim-up and the first 2 h of embryo culture did not improve cleavage rates, and was detrimental to further embryo development.

Keywords: Equine, oocytes, intracytoplasmic sperm injection, embryo culture

Acknowledgements

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Sperm motility and fertility of cooled preserved stallion semen in either INRA96 or EquiPro CoolGuard extender

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Semen extenders are added to stallion semen when processed for preservation under cooled conditions to aid in viability and to maintain its fertility over time. Increased duration of sperm quality and fertility potential of cooled semen is desirable because of the ease of use for breeders and veterinarians. Previous studies suggest that that the chemically-defined extender INRA96 (IMV Technologies USA, Maple Grove, MN) is superior to most milk-based extenders when ejaculates are incubated at 4°C for up to 48 hours. EquiPro CoolGuard (Minitube of America, Verona, WI) is a new commercial semen extender that is defined specifically to maintain quality of semen and fertility after preservation at 4°C. The objectives of this study were to: a) evaluate the motility of stallion spermatozoa after cooling for 0, 24, 48, 72, and 96 hours in both INRA96 (INRA) and EquiPro CoolGuard (CG), and b) observe the fertility rates of a small group of mares inseminated with semen extended with CG at either the day of collection or 96 hours post-collection after cooled preservation in CG. We hypothesized that CG would record similar motility data to that of INRA, but that fertility rates would decrease after cooled storage of sperm for 96 hours. For the first experiment, semen was collected daily for three days from six stallions (n=6) using a Missouri model artificial vagina, after having been collected for a week prior to the experiment. Ejaculates were centrifuged at 600g to reduce the seminal plasma to 90% and reconstituted in INRA and CG, respectively, at a concentration of $20x10^6$ spermatozoa/mL. Samples were stored in an Equitainer® (Hamilton Thorne, Beverly, MA) to be gradually cooled to a final resting temperature of 4°C. Total motility and progressive motility were recorded via a CASA system (SpermVision, Minitube of America) at 0, 24, 48, 72, and 96 hours after collection. For the second experiment, nineteen mares were bred with $1x10^9$ sperm pooled from two stallions in 10mL CG either 6 hours after collection (n=11) or at 96 hours after collection (n=8). Statistical analysis for the first experiment was done via a two-way ANOVA on repeated measures with significance set to $P<0.05$. While total and progressive motility decreased over time, no significant differences were found between the two extenders for either endpoint at any time. Both extenders demonstrated support of semen up to 96 hours with average progressive motility being approximately 40% at the end of the experiment. Eight of the eleven mares (72.7%) who were bred with CG on the day of collection and six of the eight mares (75%) who were bred with CG 96 hours after collection were pregnant as determined by ultrasonagraphic evaluation at 24 days after insemination. In conclusion, similar motility data were observed for both extenders, demonstrating the ability to maintain acceptable sperm viability for up to 96 hours after collection. Recognizing that only a small number of mares were bred in this study, we also conclude that fertility rates were comparable to the rates seen in both natural breeding as well as artificial insemination using fresh semen when semen was extended with CG for 96 hours at 4°C.

Keywords: Extender, INRA96, CoolGuard, fertility, motility

Disclosure

M.H.T. Troedsson is affiliated with Minitube of America as a consultant.

Urethral diverticulum in a breeding stallion

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A urethral diverticulum is a localized saccular dilation of the urethra that communicates to the urethral lumen, and it can be classified as either congenital or acquired. In male horses, urethral defects, such as urethral rents, are common and occur most often on the caudodorsal convex surface of the urethra at the level of the ischial arch. However, an acquired urethral diverticulum due to a breeding injury has not been previously described. A six year-old AQHA stallion was presented in December of 2010 with a prior history of a breeding injury involving the penis and a surgical intervention to repair his injured urethra 1.5 years previously. The stallion had a successful breeding a year after surgery. The stallion was referred due to blood dripping from the penis after a breeding attempt. The stallion had normal physiological parameters, the left testis was 9.0 x 5.5 x 7.5 cm (L x W x H) with an echo dense region (0.6. x 0.6 cm) on the caudoventral aspect of the testis, lacking blood flow (color Doppler), and was interpreted as a calcification. The right testis was located in the inguinal area and measured 6.4 x 3.0 x 3.0 cm (L x W x H). The gross external examination of the penis and ultrasound examination of the accessory sex glands revealed no abnormalities. Semen collection was attempted. The stallion showed good libido, but stopped thrusting prior to ejaculation, and blood was noted coming from the urethra. A urethroscopy was performed and revealed urethritis, small ulcers, mucus material in the urethra and bladder. In addition, a dorsal urethral diverticulum, 50 cm from the urethral opening, which communicated with the corpus spongiosum was noted. The tissue of the diverticulum was severely inflamed, and coated with mucoid material and urine crystals. Culture and sensitivity of the different urethral lesions and bladder yielded >2+ *Streptococcus zooepidemicus*. Antibiotic therapy with trimethoprim sulphonamide (TMS; 30mg/kg) SID for three weeks, sexual rest, followed by reassessment for surgery was recommended. At the subsequent examination, the effected urethral tissues appeared less inflamed and culture revealed no growth. Penile ultrasound then showed a 2 x 3 cm linear diverticulum that communicated with a larger fistulous diverticulum. An ejaculate was obtained and semen analysis showed 43% morphologically normal sperm, with low motility. Antimicrobial therapy was discontinued for three days and a urine sample and cytobrush swab from the urethral diverticulum yielded *Streptococcus equi zooepidemicus* only from the urethral diverticulum. A perineal urethrostomy and distal urethrostomy over the diverticulum was performed with placement of a Penrose drain which was left in place for 12 days. Treatment included phenylbutazone, TMS BID for 16 days, with daily lavage using lactated Ringer's solution followed by 8 mM TRIS-EDTA for 10 days. The incisions were left to heal by second intention. The TRIS-EDTA was used to treat the urethra and diverticulum as a pathologic biofilm was suspected. The surgical lesions healed and the stallion was retired from breeding. A buccal mucosal urethroplasty has been successfully used as a treatment for a urethral rent and was considered in this case.

Keywords: Urethral diverticulum, urethritis, urethroscopy

Incidence rate and effects of persistent mating-induced endometritis (PMIE) in Quarter Horse mares

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The goal of this retrospective study was to determine if a relationship exists between mare age and accumulation of intrauterine fluid after breeding and if this fluid affects pregnancy rates in a clinical setting. The hypotheses were: a) older mares will have a greater incidence of intrauterine fluid retention 24 hours after breeding and b) a reduction in pregnancy rates will occur in mares that retain intrauterine fluid for greater than 24 hours after breeding.

Mares were managed at the Equine Reproduction Laboratory at Colorado State University. Inclusion criteria for the study were as follows: Quarter Horse mares, ovulation within 48 hours of breeding, and pregnancy or embryo flush result recorded. Treatments for intrauterine fluid were performed at the discretion of the attending clinician. Data were compared by a contingency table utilizing Fishers Exact test. Data were presented as the mean +/- SD.

The overall incidence of PMIE was 36%. A significant ($p<0.05$) effect of age was observed on the incidence of fluid >24 hours (Table). A significant reduction in pregnancy rates was observed in mares that had intrauterine fluid 48 hours after insemination compared to mares with normal or no fluid (34% vs 60%). There was no effect of intrauterine fluid on pregnancy rates within age groups (Table). There was a trend ($p=0.052$, 0.054, respectively) for an effect of age on conception rates for mares 16-20 and >21 years of age.

In conclusion, older mares (≥ 16 years of age) have an increased incidence of PMIE subsequent to insemination and should be monitored closely following breeding. If intrauterine fluid is present at 48 hours after breeding, a reduction in pregnancy rates is observed.

Mare Age	No Fluid 24 hrs after AI		Fluid 24 hrs after AI		Fluid 48+ hrs after AI	
	Incidence	Pregnancy Rate	Incidence	Pregnancy Rate	Incidence	Pregnancy Rate
≤5 years (n=18)	100%[a] 18/18	77.7%[a] 14/18	0%[a] 0/0	N/A	0%[a] 0/0	N/A
6-10 years (n=80)	76.2%[b] 61/80	68.9%[a] 42/61	18.75%[a] 15/80	73.3%[a] 11/15	5%[a] 4/80	25%[a] ¼
11-15 years (n=93)	60.2%[c] 56/93	58.9%[a] 33/56	29%[b] 27/93	62.9%[a] 17/27	10.8%[a] 10/93	50%[a] 5/10
16-20 years (n=58)	50%[c] 29/58	51.7%[a] 15/29	32.8%[b] 19/58	57.9%[a] 11/19	17.2%[a] 10/58	30%[a] 3/10
≥21 years (n=21)	42.9%[c] 9/21	44%[a] 4/9	19.1%[b] 4/21	75%[a] 3/4	38%[b] 8/21	25%[a] 2/8

Table. Incidence rate of intrauterine fluid 24 and 48 hours after artificial insemination. Difference in superscript[a,b,c] within a column represents a significant ($p<0.05$) difference.

Keywords: Conception rate, equine, intrauterine fluid, persistent mating-induced endometritis

Dehydroepiandrosterone sulfate and testosterone concentrations in mares carrying normal pregnancies

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Dehydroepiandrosterone, a product of the fetal gonads, is utilized by the placenta as a precursor for estrogen synthesis during pregnancy. Dehydroepiandrosterone is sulfo-conjugated by the fetal-placental unit. In mares, circulating testosterone is elevated during pregnancy, and this elevation has been associated with the fetal-placental unit. As pregnant mares have increasing concentrations of circulating estrogens between 210 and 240 days of gestation, we hypothesized that androgens achieve maximal concentrations at similar gestational age. The objective of this study was to describe dehydroepiandrosterone sulfate and testosterone concentrations in mares carrying normal pregnancies. Eighteen light-breed mares were used in this study. Mares were bred with fresh extended semen during the breeding season of the northern hemisphere. Ovulation was determined by transrectal ultrasonography by examinations carried out every other day. Blood samples were collected every two weeks from 100 days of gestation to term. Blood was allowed to clot, and serum was harvested and preserved at -20°C until further analysis. Postpartum placentas were examined to assure normality. Determination of dehydroepiandrosterone sulfate (CV's; intrassay 6.5% and interassay 10.3%) and testosterone (CV's; intrassay 9.7% and interassay 17%) were achieved with specific equine immunoassays. Data were log-transformed and analyzed using a mixed model (with mare as random effect and time as a fixed effect). Post hoc comparisons were made by Fisher's protected least significant difference (day effect) test. Significance was set at $p<0.05$. All mares had normal gestation lengths (mean 348 ± 2.8 days) and delivered normal placentas. There was significant day effect for both androgens ($p<0.0001$). Concentrations of dehydroepiandrosterone sulfate peaked by six months of gestation ($p<0.05$), which is one month before the reported peak for estrogens. However, testosterone was progressively elevated from days 100 to 180 ($p<0.05$), and then plateaued until ~240 days; thereafter a progressive decline was observed until 290 days of gestation ($p<0.05$). Concentrations of testosterone remained constant between 290 days and term ($p>0.05$). In conclusion, dehydroepiandrosterone sulfate and testosterone concentrations were elevated in pregnant mares and varied through day 100 to term. As estrogens have been extensively used as biomarkers for pregnancy loss in mares, androgens may be useful biomarkers in mares suffering placental disease; however, this hypothesis should be addressed in further studies.

Keywords: Pregnancy, androgens, fetal-placental unit, fetal gonad, horses

Acknowledgments

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The influence of the hormonal environment and a bacterial challenge on global gene expression in the equine uterus

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As the uterus adapts to meet its multiple reproductive functions, it is strongly influenced by ovarian hormones. Our hypothesis is that these influences modulate the ability of the uterine innate immune system to respond to bacterial challenges, and as such can potentially be used as a model for persistent mating-induced endometritis (PMIE). Hence, a global gene expression analysis of these immune changes could help find targets for the development of new treatment approaches.

The objectives of this study were: a) to generate normal uterine gene expression profiles in clinically healthy horses during different stages of the estrous cycle; and b) to characterize differences in gene expression patterns in endometrial tissues following intrauterine bacterial pathogenic inoculation.

Five Standardbred mares (3 to 4 years old, shown to be resistant to PMIE) were inoculated with an *E. coli* strain isolated from a mare susceptible to PMIE. They were inoculated once during estrus and once during diestrus. The absence of inflammatory signs was confirmed between treatments. Biopsies were obtained before and three hours after the inoculation. For each tissue sample, total RNA was isolated (Life Technologies, Mulgrave, Australia), cDNA libraries were constructed (TruSeq, Illumina, San Diego, CA), and paired-end RNA-Seq data sequenced (HiSeq, Illumina). High quality sequence reads were mapped to the annotated Ensembl horse genome (version 71), then gene transcription was inferred. For each treatment, normalized gene transcription values were used to hierarchically cluster genes. Clear gene clusters were then identified in which gene expression levels differed between the cycle stages as well as in the pre- and post-inoculation samples. Further analysis using the KEGG database (http://www.genome.jp/kegg/pathway.html) linked several pathways associated with the innate immune system to differentially clustered genes. Subsequently, differentially transcribed genes were identified using the Cufflinks package.

In total, 4430 genes were differentially expressed ($P<0.05$) between estrus and diestrus in clinically healthy horses. In response to a bacterial challenge, the expression of 6782 genes was altered ($P<0.05$) in estrus, whereas 2225 genes were differentially expressed ($P<0.01$) after the inoculation with *E. coli* in diestrus.

The results of this study reveal significant changes in expression associated with differences in the ovarian hormone environment during different cycle stages. Furthermore, a significantly greater number of genes were differentially expressed three hours after a bacterial challenge during estrus than during diestrus. Analyses now in progress will further functionally characterize the differentially regulated genes to extract specific information about the altered cellular pathways, in particular those related to immune function.

Keywords: Horse, uterus, RNA-Seq, innate immunity, *E.coli*

Effect of frozen-thawed granulosa cells and serum additives on individual bovine embryo development

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Individually cultured bovine embryos have shown a reduced ability to proceed through development to the blastocyst stage when compared to cultures containing multiple embryos. The origin of this deficit is not entirely clear, although it is suspected to be due to a dilution of autocrine/paracrine factors secreted by the zygotes. Individual cell culture is sometimes necessary, as in the case of low oocyte numbers, or when individual embryo identification is needed. Thus, the aim of this project was to evaluate the effects on individual bovine embryo development of adding granulosa cells and/or two types of serum to the culture media. Our hypothesis was that addition of granulosa cells, 10% fetal bovine serum, knockout SR serum replacer, or a combination of serum and cells would improve development to the blastocyst stage of individually cultured bovine embryos up to the level of development seen in group culture. Ovaries were obtained from a local abattoir and all visible follicles aspirated. The recovered oocytes were washed, evaluated and selected for integrity of cumulus cell layers, and individually matured in maturation medium for 23±1 h. Oocytes were then moved to individual fertilization droplets and 25,000 frozen-thawed motile sperm were added to each droplet and co-cultured for 18 h. Then, the presumptive zygotes were randomly divided into six treatment groups with a 3x2 design. A culture of frozen-thawed mitomycin-c treated bovine granulosa cells was established in the bottom of droplets in half of the treatment groups. Both groups, with and without granulosa cells, were further divided into three serum treatment groups. The embryo culture media (synthetic oviductal fluid) was altered by the addition of 10% fetal bovine serum, Knockout SR serum replacer, or not modified. Embryos were assessed on day 8 post-fertilization to determine blastocyst development, and on days 10-11 post-fertilization to evaluate hatching success. A separate group of oocytes was matured, fertilized, and cultured in a group (n=10) as a standard with which to compare the treatment groups. The results showed that none of the blastocyst rates of the six treatment groups were statistically different from each other (avg. 4.7%), with the group culture being significantly higher than all of them (21.7%) (one-way ANOVA). When aggregated, none of the interventions had a significant effect on blastocyst rate in individual culture. However, a trend (p=0.06) was found in the hatching rate between blastocysts cultured in media including cells (71.4%) and those cultured without cells (18.2%). This suggests that although the addition of granulosa cells did not increase the number of blastocysts formed, it increased the health and viability of the ones which did form.

Keywords: Bovine, embryo, individual culture, granulosa

Fertility in goats during the non-breeding season using a short-term progesterone priming protocol with either GnRH or hCG to induce ovulation

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The purpose of this project was to compare ovulation and pregnancy rates in goats bred during the non-breeding season using a short-term progesterone (P4) priming protocol with either gonadotropin releasing hormone (GnRH) or human chorionic gonadotropin (hCG) to induce ovulation. While GnRH acts on the pituitary to produce luteinizing hormone (LH) that stimulates ovulation, hCG elicits ovulation by binding directly to LH receptors on the ovary. Our hypothesis was that during the non-breeding season using a hormone that acts at the level of the ovary (hCG) would increase ovulations and pregnancy rates compared to a hormone that acts at the level of the hypothalamus/pituitary (GnRH).

Fourteen mixed breed Alpine and Saanen dairy goats were assigned to Group 1 (GnRH, n=6), Group 2 (hCG n=6), or controls (n=2). Progesterone was administered using a controlled internal drug releaser (Eazi-Breed CIDR Sheep & Goat; Pharmacia and Upjohn, New York, NY) inserted into the vagina for three days. Follicle stimulating hormone (Follitropin-V; Bioniche, Belleville, Ontario, Canada) was administered once on the second and third days of P4 priming (32 mg IM). Prostaglandin F2α (PGF2α; Lutalyse; Pfizer, New York, NY) was given (5 mg IM) on the day of CIDR removal. Two days after CIDR removal, Group1 received 50 mcg IM of GnRH (Cystorellin; Merial, Duluth, GA), while Group 2 received 500 IU IM of hCG (Chorulon; Intervet, Millsboro, DE). Control animals received only CIDRs and PGF2α as the other groups. All does were naturally bred by the same buck at the first sign of estrus and every twelve hours thereafter until they were no longer receptive. Blood was drawn daily until 26 days after breeding to determine serum P4 levels using radioimmunoassay (Coat-A-Count Progesterone RIA kit; Diagnostic Products Corporation, Los Angeles, CA).

Although the trend was for more animals to ovulate and become pregnant in the hCG group, the numbers were too small for a meaningful interpretation of the outcome.

Group	% In Estrus	% Bred	% Ovulated*	% Pregnant
1 (GnRH n=6)	100 (6/6)	33.3 (2/6)	50 (3/6)	16.7(1/6)
2 (hCG n=6)	100 (6/6)	100 (6/6)	83.3 (5/6)	50 (3/6)
Control (n=2)	0 (0)	0 (0)	0 (0)	0(0)

*Ovulation was determined by measuring serum P4.

In conclusion, hCG is as effective as GnRH in producing ovulations and subsequent pregnancies as part of a short-term P4 priming protocol used during the non-breeding season. Studies with more animals are needed to determine if hCG can actually increase fertility when compared to GnRH.

Keywords: Non-breeding season, hCG, GnRH, estrous synchronization, goats

Reproductive performance following long- vs short-term progesterone-based synchronization protocols for fixed-time artificial insemination in beef heifers

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The objective of this study was to determine the effect of long-term (CPG group; 14-d controlled internal drug release (CIDR)-prostaglandin (PG)-gonadotropin releasing hormone (GnRH) or short-term (FCC group; 5-d CO-Synch+CIDR) progestin-based synchronization protocols on artificial insemination (AI) pregnancy rate in beef heifers. The hypothesis was both long and short-term progesterone based protocols would result in similar AI pregnancy rates. Angus cross beef heifers (N = 1887) at nine locations were included in this study. All heifers received a body condition score (BCS) and reproductive tract score (RTS) and within the herd were randomly assigned to CPG or FCC protocol groups. Heifers in CPG group received a CIDR (1.38 g of progesterone; Eazi-Breed CIDR Cattle Insert®, Pfizer Animal Health, New York, NY) from day 0 to 14, followed by 25 mg of dinoprost (Lutalyse®, Pfizer Animal Health) 16 days later (day 30). Heifers in FCC group received a CIDR and 100 µg of gonadorelin hydrochloride (Factrel®, Pfizer Animal Health) on day 0 (day 25) followed by 25 mg of dinoprost at CIDR removal and a second dose of dinoprost, (25 mg), 6 h later on day 5 (day 30). Artificial insemination was performed at 72 h (day 33) for CPG group and at 56-h for FCC group (day 32) after CIDR removal, and all heifers were given GnRH (100 µg, IM) concurrently at the time of AI. Two weeks later, intact bulls were placed with the heifers for the remainder of the 60-70 d breeding season. Heifers were examined for pregnancy status 50 to 70 days after AI to determine if pregnancy was the result of AI or bull breeding.

The data were analyzed using mixed model procedure (PROC GLIMMIX SAS). The variables included in the model were treatment (CPG vs. FCC), BCS categories (4, 5 and 6, and 7), RTS (≤3, 4 and 5), treatment by RTS categories and treatment by BCS categories interactions. Year (location), AI sires and AI technicians were included as random variables in the model. The P value was set at > 0.1 for exclusion and $\alpha \leq 0.05$ for significance. No difference in AI pregnancy rates between CPG and FCC synchronization protocols [54.5% (489/897) 55.5% (549/990); (P=0.92)] was observed. The AI pregnancy for heifers with RTS ≤3, 4 and 5 were 52.6, 53.6 and 59.9% respectively (≤3 vs 5 P<0.05). The BCS, treatment by RTS categories and treatment by BCS categories interactions did not influence AI pregnancy (P>0.1). In conclusion, heifers synchronized for fixed time AI with 14-d CIDR-PGF-GnRH and 5-d CO-Synch+CIDR resulted in similar AI pregnancy rates and thus the short-term protocol offers an additional opportunity for open heifers to become pregnant.

Keywords: Beef heifers, CIDR, PGF, estrous synchronization, insemination; pregnancy

Evaluation of effects from chlorhexidine hydrochloride intrauterine suspension administration in normal mares

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Introduction

Chlorhexidine gluconate solution could be used effectively for uterine lavage, but historically this substance has been implicated as irritating to mucous membranes, including the endometrium of the mare. We evaluated the use of chlorhexidine hydrochloride suspension (Nolvasan® Suspension, Zoetis, Florham Park, NJ) in the uterus of normal mares to determine if adverse effects on endometrial health were noted. We hypothesized that administration of chlorhexidine hydrochloride intrauterine suspension would result in endometrial inflammation or changes in Kenney biopsy score grades in treated mares relative to placebo treated controls.

Methods

Twelve healthy, adult light breed mares were used for this study. All procedures were approved by the Auburn University Institutional Animal Care and Use Committee. All mares were determined to be reproductively normal prior to inclusion in the study by evaluation of endometrial histopathology, cytology, and bacterial culture. Mares were randomly assigned to the treatment group or control group (n=6 per group). Each mare was treated during estrus with an intrauterine infusion of 1 gm (28 mL per tube; 35.7 mg/mL) of chlorhexidine hydrochloride suspension (treatment group) or an equal volume of lactated Ringer's solution (control group) once daily for three consecutive days. Biopsy and cytology samples were taken 3, 7, and 14 days after completion of treatment. Cytology and biopsy samples were read by a board certified pathologist (LN) blinded to the treatments, and biopsy samples were graded using a standardized Kenney score.

Results

Differences between groups were assessed with Fisher's exact test using commercially available statistical software (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). There was no difference with respect to Kenney grade biopsy score, degree of endometrial fibrosis, or presence of cytologic inflammation between control and treatment groups ($p=0.55$, 0.7, and 0.06, respectively); no difference was detected when accounting for sampling day. The suspension was grossly visible in the uterine lumen when mares were examined with transrectal ultrasonography for up to four days after treatment.

Conclusions

Based on analysis of endometrial biopsy scores, degree of fibrosis, and presence or absence of inflammation, there was no difference between treated and control mares. Treatment with chlorhexidine hydrochloride at this concentration does not appear to have a deleterious effect on short term endometrial health in mares.

Acknowledgement

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Keywords: Chlorhexidine, endometrium, fibrosis

A split-double intramuscular administration of Folltropin®-V with hyaluronan - a new approach to superovulation for embryo flushing in ewes

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Traditional superovulatory protocols in ewes consist of twice daily IM injections of follicle stimulating hormone (FSH) for four days. Although this is an efficient method, the use of a simple regimen with minimum number of treatments is desirable from the labor and animal welfare perspectives. Folltropin®-V is the common FSH preparation used for superovulation. One of the ways the number of Folltropin®-V treatments can be reduced is by diluting the agent in a slow-release hyaluronan-based formulation (MAP®-5; Bioniche Animal Health, Bogart, GA). This model has been developed and successfully used in beef cattle. In December 2013 an experiment was conducted using Dorset-based whiteface cross ewes with an average age of four years. The ewes were placed on pasture, fed grain diets, and offered free choice mineral during the study. The ewes were selected randomly and separated into two groups. Group I (Control; n=9) ewes received the traditional treatment (252 mg) shown in the table while Group II (hyaluronan; n=8) ewes received the two dose treatment of Folltropin®-V diluted in MAP-5 (168 mg and 84 mg, respectively, 48 hours apart) on days 12 and 14. Ewes were inseminated via laparoscopic intrauterine insemination and surgicallyflushed for embryo recovery. The total number of corpora lutea and embryos were determined. Embryos were classified using the International Embryo Transfer Society evaluation standards. For both Groups the average number of corpora lutea and the number of fertilized embryos were 8 and 7 respectively. The parameters evaluated between the Groups were not different ($P > 0.05$). The findings suggest that the superovulatory response and the quality of embryos were similar in multiple Folltropin®-V and a split-double intramuscular administration of Folltropin®-V with hyaluronan treatments. The findings merit further evaluation with a larger sample size.

Day 1	Insert CIDR
Day 7	Change CIDR + 250 µg cloprostenol
Day 12	AM –50 mg Folltropin®-V PM – 50 mg Folltropin®-V
Day 13	AM –32 mg Folltropin®-V PM – 32 mg Folltropin®-V
Day 14	AM –24 mg Folltropin®-V PM – 24 mg Folltropin®-V + 200 IU equine chorionic gonadotropin (Folligon; Merck Animal Health, Kirkland, QC, Canada) and remove CIDR
Day 15	AM –20 mg Folltropin®-V and remove feed/water. Teasers added to pen PM – 20 mg Folltropin®-V
Day 16	AM – Laparoscopic intrauterine insemination with fresh semen
Day 21	AM – Remove feed/water
Day 22	AM – Embryo Flush

Keywords: Ewes, superovulation, Folltropin®-V, MAP®-5, embryo

Acknowledgement

Supply of the compounds used in the study was kindly provided by Dr. J. Denton of Bioniche Life Science Inc., ON, Canada.

Deep-horn intra-uterine application of prostaglandin E_2 does not hasten oviductal transport of equine embryos

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Intra-luminal application of prostaglandin (PG) E_2 to the equine oviduct on day 3 after ovulation hastens embryo transport into the uterine lumen by inducing the same physiological response as the endogenous embryonic signal of selective oviductal transport.[1] More recently, laparoscopic application of PGE_2 to the surface of the oviduct has been used as a treatment for oviductal blockage in mares with unexplained infertility.[2,3] Although it is apparently effective for restoring oviductal patency, laparoscopic application is invasive and requires specialized surgical equipment. Therefore, development of more practical methods of locally administering PGE_2 to the oviduct would be useful. We hypothesized that deep-horn intra-uterine application of PGE_2 would hasten oviductal transport, serving as a "proof of principle" for using that route of delivery to restore oviductal patency. Therefore, the objective of this study was to determine if deep-horn intra-uterine application of PGE_2 on day 3 after ovulation would hasten oviductal transport. Sixteen mares, 2 to 12 years old, were examined daily with transrectal palpation and ultrasonography until a follicle $\geq$35mm and prominent uterine edema were present, at which time they were inseminated with at least 500 million progressively motile spermatozoa and administered an ovulation-inducing agent; 10 mares (5/group) received 1.8 mg deslorelin acetate IM (SucroMate™ Equine, Thorn BioScience LLC, Louisville, KY) and 6 mares (3/group) received 2,500 IU hCG IV (Chorulon®, Intervet/Merck Animal Health, Summit, NJ). Daily examinations were continued until the day of ovulation (day 0). Mares that did not ovulate within 48 hours of breeding were inseminated again. On day 3 mares were randomly assigned to receive 0.5 mg PGE_2 (Prepidil® Gel; Pfizer Inc., NY, NY) or an equal volume (2.5mL) of vehicle (240 mg colloidal silicon dioxide and 2.76 g triacetin) via deep-horn administration ipsilateral to ovulation. Twenty-four hours later (i.e., on day 4), embryo collection was performed using 4 L of flush medium and the efflux was examined for embryos and/or unfertilized oocytes. No embryos or oocytes were recovered from any PGE_2-treated (0/8) or vehicle-treated (0/8) mares. When mares were examined for pregnancy on days 11 to 17, 2/8 (25%) PGE_2-treated and 4/8 (50%) vehicle-treated mares were confirmed pregnant. These results indicate that deep-horn intrauterine application of PGE_2 does not hasten embryo transport, and therefore cannot be advocated as a treatment for oviductal blockage in mares.

Keywords: Equine, mare, oviduct, PGE_2, embryo

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Comparison of cauda epididymal sperm morphology following surgical vasectomy or chemical epididectomy in feral horses

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Introduction

Following surgical vasectomy or ductal occlusion, epididymal distension with changes to the interstitium occur.[1] In laboratory animals, pressure-mediated damage to the seminiferous epithelium can also follow.[2] Surgical vasectomy and chemical epididectomy are two methods under investigation for use to control feral horse populations. The objective of this study was to determine if either method induced significant changes in spermatogenesis. The hypothesis was that surgical vasectomy and chemical epididectomy would increase the percentage of abnormal sperm present in the tail of the epididymis.

Methods

Stallions used in this study were part of the feral horse herd managed in accordance with the U.S. Fish and Wildlife Service comprehensive conservation plan for the Sheldon National Wildlife Refuge. Stallions were either surgically vasectomized (n=9), chemically epididectomized (n=11) or untreated (n=11) in the five years prior to this study. For the chemical epididectomy, 10 ml of 1% chlorhexidine in 90% DMSO (v/v) was injected into the tail of each epididymis. In 2013, stallions were gathered for routine castration. Immediately following castration, testes, epididymides and vas deferens were examined and any abnormalities found were noted. Next, a sample of fluid from the tail of each epididymis was mixed with eosin-nigrosin morphology stain, spread with a spreader slide, and allowed to air dry. The slides were evaluated blindly under oil immersion (1000X) without a coverslip using bright field microscopy and the percentage of sperm with abnormal morphology was determined. The effect of treatment on sperm morphology was compared using a one-way ANOVA test. Statistical tests were performed using Graph Pad Prism (LaJolla, CA). Significance was defined as $p<0.05$.

Results

Both surgically vasectomized males (64.8±7.8%) and the males receiving a chemical epididectomy (53.6±10.7%) exhibited significantly more abnormal sperm than normal males (40.3±4.6%; $p<0.05$). In addition, the number of abnormal sperm in vasectomized males was significantly greater than the number in the chemically treated males ($p<0.05$). The majority of the defects recorded in all groups were detached heads, bent tails, and coiled tails.

Discussion

Both surgical vasectomies and chemical epididectomies in stallions lead to significant increases in abnormal sperm and have potential for use in controlling feral horse populations. Histopathology studies are underway to determine the etiology of the observed effects on spermatogenesis.

Keywords: Epididectomy, equine, sperm morphology, vasectomy

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Uterine artery blood flow and arterial diameter correlate closely with gestational age in the mare

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Determining fetal age in equine pregnancy is complicated by maternal size and variations in fetal growth-rate. Previous studies have correlated fetal organs, heart rate and ocular diameter with gestational age. To date, no model has achieved sufficient predictive values for clinical application. More recently, the blood flow parameters resistance index (RI) and pulsatility index (PI) have been correlated broadly with gestational age.[1] In the current study, we hypothesized that uterine artery diameter (D) and total arterial blood flow (TABF) would be highly correlated to gestational age in mares.

A total of 149 ultrasound examinations in nine healthy pregnant pony mares were performed between 35 and 335 days of gestation, using a 5-7 MHz linear probe with Doppler capability (Sonosite MicroMaxx™, Bothell, WA). Mares were examined irregularly at intervals ranging from 24 hours to weekly, as dictated by other ongoing studies. Diameter, maximum flow-velocity (Vmax), RI, and PI from each day were measured in triplicate, averaged for both the right and left uterine arteries, and were used to calculate TABF as previously described.[2]

Mares were grouped in two weight-categories (350 to 450 kg, and 450 to 550 kg). Data were analyzed for effect of gestational age using logarithmic (TABF) or linear (D) regression. Total arterial blood flow and D were both highly correlated with gestational age for mares of similar body-weight (350 to 450 kg: D: $r^2 = 0.902$, TABF: $r^2 = 0.905$; 450 to 550 kg: D: $r^2 = 0.891$, TABF: $r^2 = 0.905$; $p<0.0001$ for all values). These results suggest that uterine artery measurements may be useful in the prediction of gestational age in mares.

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Keywords: Equine, Doppler ultrasound, gestational aging, blood flow

Diagnostic double guarded low-volume uterine lavage in mares

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Endometritis constitutes a major problem in the management of broodmares; hence diagnostic tests with a high sensitivity and specificity are desired. We hypothesize that a double guarded uterine flush technique for bacterial culture and cytology is comparable to standard diagnostic tests, the endometrial biopsy and double guarded swab.

Endometrial biopsies (n=199), swabs (n=199) and double guarded lavage samples (n=199) were obtained from 34 mares at six different time points in four estrous cycles, and were evaluated cytologically and bacteriologically. Endometrial biopsies from the first cycle (n=34) were examined for the presence of polymorphonuclear neutrophils (PMNs) in the endometrial epithelium and was used as a gold standard for calculation of diagnostic sensitivity.

E. coli was most frequently isolated (lavage: 31%, swab: 21%, biopsy: 12%) followed by β-hemolytic streptococci (lavage: 11%, swab: 8%, biopsy: 7%) (positive bacterial growth > 4 colony forming units (CFU)). Positive cytology was less likely to occur when *E. coli* was isolated from the diagnostic tests compared to the growth of β-hemolytic streptococci. Isolation of pathogens from uterine samples was highly associated with the presence of PMNs on histology (p=0.003). Using the presence of PMNs in the endometrial tissue as the gold standard for diagnosing endometritis, the sensitivity of double guarded lavage culture was 0.75, and 0.33 and 0.5 for the swab and biopsy, respectively.

In conclusion, the double guarded lavage technique was an accurate method for diagnosing mares with endometritis and the risk of false positive samples is considered to be minimal compared to other flushing techniques described.

Keywords: Endometritis, double guarded lavage, diagnostic test, *E. coli*, β-hemolytic streptococci

Effects of a third-generation GnRH antagonist on reproductive parameters in the stallion

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Numerous investigators have attempted to suppress the hypothalamic-pituitary-gonadal axis in the stallion with varying results. We **hypothesized** that the gonadotropin releasing hormone (GnRH) antagonist acyline would lead to such down-regulation. The objective of the current study was to evaluate the effect of acyline on testis volume, peripheral testosterone concentration, seminal parameters and reproductive behavior in stallions. Stallions (n=4) were treated (330 µg/kg acyline IM every 5 days) for 57 days, and vehicle-treated stallions (n=4) served as controls. Semen was collected and evaluated daily on alternate weeks; time to erection and ejaculation were recorded; and testis volume was measured weekly by ultrasonography. Serum testosterone concentrations were measured by EIA. Data were analyzed using a random-effects mixed model. There was a significant time by treatment interaction ($P<0.05$) on serum testosterone concentration (Day 3), testis volume (Week 5), total sperm number (Week 1), and total sperm motility (Week 2); with the first significant decline in each parameter noted in treated stallions in parentheses. There was no effect of acyline treatment on time to erection or ejaculation. Following cessation of acyline treatment, serum testosterone concentrations, testis volume, total sperm number and sperm motility appeared to recover their pretreatment values. Overall, this study demonstrates that administration of acyline to the stallion results in a rapid suppression of the hypothalamic-pituitary-gonadal axis with recovery following cessation of treatment. Interestingly, measured behavioral parameters were not different in treated stallions compared to controls.

Acknowledgments

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Keywords: Testosterone, GnRH antagonist, acyline, stallion.

Sudden infertility of a stallion collected with a commercial artificial vagina

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Importance

Sudden infertility in stallions from one breeding season to the other may occur due to age, trauma, or disease. However, when semen collection is performed with a consistent protocol and equipment, sudden infertility is not expected. In this case semen collection and processing were performed using the same protocol as the previous 18 years. A new artificial vagina (AV) was assigned to the stallion without any decrease in semen quality and quantity when examined immediately following collection, but no pregnancies resulted from breedings.

Diagnostic approach and treatment

A 23 year old Tennessee Walking Horse stallion with a history of normal fertility during the previous breeding season was presented for sudden infertility. No pregnancies resulted when mares were artificially inseminated (AI) with extended fresh semen collected with a commercial AV. A complete breeding soundness examination revealed no abnormalities consistent with infertility. Evaluation of concentration, motility and morphology of the semen immediately following collection was within normal limits and sufficient to produce pregnancy (concentration 150-190 M/ml, motility 50-70%, morphology 60-71%). Subsequent evaluation of fresh and chilled extended semen was performed at 5-15 minute intervals following collection. The motility of collected samples decreased rapidly and reached 0% within an hour of collection. Multiple variables were individually adjusted in attempts to identify potential spermatotoxins. Improving temperature control of collected samples, use of various extenders, removal of the seminal plasma and AV were all investigated.

Result and discussion

The rubber of a specific new AV used solely for this stallion at the beginning of this breeding season was determined to be the cause of sperm death. Semen collection was performed with a liner in the AV and resulted in 60% motility when chilled semen was evaluated 48 hours following collection. Additionally, the same AV was used without a liner to collect other stallions and resulted in motility decreasing to 0% within an hour.

Stallions are usually collected with a commercial AV when semen is to be used for AI. Artificial vaginas are generally cleaned according to the manufacturer's indications, but disposable liners are rarely used when the AV is allocated to a particular stallion for the breeding season. Changes in the material used to make a new AV can be detrimental and go unnoticed until multiple mares are diagnosed to be not pregnant.

Keywords: Semen, stallion, artificial vagina, infertility, mares.

Comparison of two pharmacologic protocols to induce ex copula ejaculation in novice stallions

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Ex copula ejaculation can be induced pharmacologically to collect semen from injured or debilitated stallions. Studies of ex copula induction of ejaculation in stallions using imipramine and xylazine have cited high response rates (up to 70%); however, in our clinic the rates have been low and highly variable (0-55%, overall 21%). One variable in success may be the sexual development and experience of the stallion. Spontaneous ejaculation in stallions sedated with detomidine (Dormosedan®, Zoetis, Florham Park, NJ) has been reported. The objective of this study was to compare two pharmacologic protocols to induce ex copula ejaculation in novice stallions, and their effects on ampullae contraction and circulating testosterone levels. The hypothesis was that imipramine and detomidine, an alpha-2 agonist with higher receptor affinity than xylazine, would result in higher rates of ejaculation compared to imipramine and xylazine. Five healthy novice (non-breeding) stallions aged 3 to 7 years were enrolled in the study to compare the following protocols to induce ex copula ejaculation: imipramine 3 mg/kg PO followed two hours later by either xylazine 0.66 mg/kg IV or detomidine 0.015 mg/kg IV. Testicular measurements and ultrasonography were performed to identify any pre-existing abnormalities, of which there were none. As the stallions were not trained to an AV, no semen collections were performed prior to enrollment. Each stallion was administered each treatment four times in a crossover design with 24 to 72 hours rest between treatments, time consistent with what is used in clinical practice. Three blood samples were collected twice for each treatment: at the time of imipramine administration, at the time of alpha-2 agonist administration, and after ejaculation or 45 minutes, whichever came first. Serum was stored at -20°C until assayed for testosterone by radioimmunoassay. Transrectal ultrasonographic measurement of the ampullae diameter was performed twice per treatment, the evening before treatment and after ejaculation or 45 minutes after alpha-2 agonist administration, whichever came first. Only one stallion ejaculated once, under the imipramine-xylazine protocol. There was no significant difference in the ampullae diameter before and after treatment for both treatments as analyzed by paired t-test ($p>0.05$). Analysis of serum testosterone by repeated measures ANOVA demonstrated no difference before imipramine treatment, before alpha-2 agonist administration, or after ejaculation or 45 minutes. Interestingly, however, there was a significant effect of treatment on testosterone, with detomidine-treated animals having higher testosterone at all three time-points ($p<0.05$). This observation remains unexplained. In conclusion, both treatments resulted in poor response of novice stallions to induction of ex copula ejaculation. Response to these treatments may be testosterone-independent. Further evaluation of factors affecting response, such as level of stress and environmental conditions, are being pursued in our laboratory.

Keywords: Imipramine, detomidine, ampullae, ultrasonography, testosterone

Longevity of chilled canine semen with Fresh Express®

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An ideal extender would preserve chilled canine semen quality for up to ten days to account for delays in ovulation in the female, transport time of semen, and availability of the male. Because of reported success of 14-day semen storage using a Tris-egg yolk-glucose extender, we examined longevity provided by Fresh Express® (Zoetis, Florham Park, NJ), a commercially available extender over a period of 14 days by examining motility, viability, and morphology of the spermatozoa and compared the results to those of a second commercially available extender. We hypothesized Fresh Express® would preserve chilled semen resulting in 60% of original motility, 75% of original viability, and negligible changes in morphology at the end of 14 days.

With an estrous bitch present, semen was collected by manual stimulation from five 18± 2 kg, 5- (±2.1) year-old, mixed breed dogs (Day 0). Initial concentration of spermatozoa (cells in 10^6/ml) and viability were determined using automated fluorescence microscopy (Nucleocounter SP-100; ChemoMetec A/S, Allerød, Denmark). The cells were centrifuged (1400 G, 10 min, 20°C) and Fresh Express® or the second extender added to adjust the concentration to 150 x 10^6/ml and each sample was split into A and B tubes. Cells were stained with eosin nigrosin and 200 cells counted to assess morphology (detached or dead heads, obvious acrosomal defects, midpiece droplets or defects, kinked, bent or coiled tails). Motility was analyzed by computer-aided sperm analysis (Hamilton Thorne, Beverly, MA) after 20 µl of each sample were diluted to a concentration of 50 x 10^6/ml. All tubes were refrigerated at 4°C for the remainder of the study. On days 3, 6, 9, 12, and 15, aliquots (A tubes) were removed from each tube, warmed to 20°C, and reassessed as described above. All B tubes were centrifuged (1400 G, 10 min, 4°C), supernatant removed, and fresh extender added before evaluation at the same time points.

Data for motility, viability and morphology were compared using Mann-Whitney statistics. After six days of refrigeration, motility of samples extended in Fresh Express® were 97.4±3.6% of the original value and statistically higher than the motility (64.1±4.6%) using the second extender. The number of viable cells after three days dropped to 91.7±4.7% and 94.7±0.9% of their original values, respectively. Morphological changes using both extenders were minimal and consisted of a less than five percent increase in detached heads and bent tails.

Keywords: Extender, longevity, canine

Evaluation of etonogestrel, altrenogest and medroxyprogesterone bioactivity in mares using an in vitro bioassay

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Many mares fail to perform at their best due to strong sexual behavior and may become harder to manage, perform irregularly, or even appear painful when in heat. Because of performance problems associated with the estrus period in mares, several different progestins have been evaluated for efficacy in suppressing estrus in the mare. Currently no single treatment has been shown to be both effective and safe for prolonged estrus suppression. Etonogestrel (synthetic progestin currently used in implant form for long-term human contraception) showed some suppression of estrous behavior in a previous field trial using human doses (2 implants/mare, Dujovne, unpublished), suggesting that the determination of an effective dose for horses may provide a novel, long-acting, alternative therapy to safely suppress heat in mares.

Dose-response studies conducted in vivo are extremely expensive. Moreover, doses cannot be extrapolated from humans; if only based on the knowledge that the binding of various presumptive progestins to equine endometrial and mammary cytosolic extracts (source of progesterone receptor, PR) differs from extracts of human endometrium. Furthermore, binding is not a reliable indicator of PR activation, receptor antagonists are effective because of high affinity binding.

The following study was conducted to investigate the relative potencies of progesterone, etonogestrel, altrenogest and medroxyprogesterone acetate (MPA) as putative activators of the equine PR.

An in vitro bioassay using Chinese hamster ovarian (CHO) cells expressing the equine PR was developed to test the relative bioactivity of various synthetic and natural progestins in horses (Scholtz et al, in press). Chinese hamster ovarian cells stably expressing the equine PR were transfected with a reporter plasmid expressing luciferase under the control of the progesterone-responsive mouse mammary tumor virus (MMTV) promoter. After 48 hrs, cells were exposed to progestins (0-1000nM) and luciferase activity was measured 24-36 hrs later.

Progesterone and altrenogest were equipotent and the most potent activators of the equine PR based on MMTV-induced luciferase expression. Almost no response was obtained with MPA even at the highest concentration tested. Etonogestrel was able to bioactivate the equine PR but at much higher concentrations than altrenogest. Therefore, the rank order of potency of these steroids as bioactive progestins in horses is predicted to be progesterone = altrenogest > etonogestrel >>> MPA.

We conclude that higher doses of etonogestrel are needed in horses than in women to induce a progestogenic response.

Keywords: Progestins, mare, estrus suppression, equine progesterone receptor, etonogestrel

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Thyroid hormone profiles in pregnant euthyroid and supplemented hypothyroid bitches

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Autoimmune thyroiditis is a hereditary disease in many dog breeds that often leads to hypothyroidism. Decreased fertility has been found in bitches with experimentally-induced hypothyroidism. In addition, decreased viability at birth, increased periparturient mortality, and reduced birth weights occur in puppies born to hypothyroid bitches. It is well documented that women with hypothyroidism require increased doses of levothyroxine during gestation to maintain a euthryoid state and deliver normal children, but this is unknown in dogs. The goal of this study was to determine if pregnant hypothyroid bitches need increased doses of exogenous levothyroxine to maintain normal plasma hormone concentrations. We hypothesized that the treated hypothyroid dogs would require increased dosages of levothyroxine to maintain a euthyroid state during pregnancy. Six bitches with experimentally-induced hypothyroidism administered levothyroxine (0.02 mg/kg q 24 h) supplementation and four euthyroid bitches were used in this study. Serum samples were collected weekly from ovulation to the end of pregnancy and assayed for total thyroxine (T4), free T4 (FT4), thyroid stimulating hormone (TSH), and 3,5,3'-triiodothyronine (T3). Supplemented hypothyroid bitches showed no clinical signs of hypothyroidism during the study and all bitches whelped normal litters. No significant changes in any of the thyroid hormones were detected during pregnancy in the euthyroid bitches. The supplemented hypothyroid bitches had elevated serum concentrations of T4 and FT4 at multiple time points during the study. These results indicate that standard levothyroxine supplementation in pregnant, experimentally-induced hypothyroid bitches is sufficient to maintain a euthryoid state. In addition, no significant changes in the thyroid hormone profiles occur in normal, pregnant bitches.

Keywords: Hypothyroidism, canine, levothyroxine, pregnancy

Effects of transvaginal ovarian biopsy on health and fertility in mares

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This is an historical perspective study from two years of data to determine the effects on health and fertility of a transvaginal ovarian biopsy (TVOB) procedure in mares. In 2012, there were 19 mares included in the study: four healthy non -TVOB mares, 11 healthy TVOB mares, and four mares with ovarian abnormalities. In 2013, there were 19 mares included in the study: four healthy non - TVOB mares, 14 healthy TVOB mares, and one mare with an ovarian abnormality. Four mares (one non - TVOB, two TVOB mares and one mare with an ovarian abnormality) were utilized in both years. Mares were classified into groups: Group 1 - non - TVOB (control n = 8 cycles); Group 2 - healthy mares one TVOB per cycle (n = 36 cycles); Group 3 - healthy mares two TVOB per cycle (n=42 cycles); Group 4 - healthy mares three TVOB per cycle (n = 5); Group 5 - mares with ovarian abnormalities (n = 5). Fertility was assessed as pregnancy rates in cycles in which mares were artificially inseminated with cooled semen from one fertile Quarter Horse stallion. Pregnancy rates were calculated for: non - TVOB Group 1 mares, the remainder of the mare cycles were further subdivided into TVOB mares that had pregnancy data before and after sampling. Pregnancy rate in mare cycles where the ovulation was from a previously biopsied ovary was 77%. Health effects were assessed with daily rectal temperatures, general demeanor and appetite for 72 hours after the procedure. There were no significant effects of TVOB on health or fertility. Histologic sections of ovaries removed from a normal mare were examined. There were no significant gross lesions. Histologic examination of the ovaries showed no fibrotic changes or architectural remodeling. Transvaginal ovarian biopsy of Group 5 mares resulted in a diagnosis of normal versus neoplastic ovarian tissue in 4/5 attempts (one sample was non-diagnostic). The TVOB may be applied to aid in the diagnosis of mares with ovarian abnormalities, and no adverse effects are to be anticipated.

Keywords: Transvaginal, ultrasound, ovary, biopsy, tumor

Table: Mare groups and pregnancy rates (PR)

Mare Groups	Number	PR
2012-2013		
Group 1 Control	8	7/8
Group 2 TVOB 1x cycle	36	11/12
Group 3 TVOB 2x cycle	42	15/16
Group 4 TVOB 3x cycle	5	1/1
Group 5 TVOB Abnormal Ovary	5	-
Total cycles	96	-
Total TVOB	116	-
Total PR TVOB mares	-	20/22

Effects of low serum progesterone concentrations on endometrial transcription at Days 8 and 12 of the estrous cycle in mares

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Changes in the endometrial transcriptome under the influence of serum progesterone (P4) have been reported in a number of species, including horses. However, there is little information available concerning relative changes in endometrial gene expression associated with low serum P4 concentrations. Our hypothesis was that low serum P4 during diestrus would affect endometrial gene expression when compared to control mares. Our objective was to evaluate changes in expression of target transcripts in mares with normal or low serum P4 concentrations. Mares were bled and scanned via rectal ultrasound on a daily basis during two cycles. Mares were randomly assigned to a uterine biopsy group (n=6 each group, on day 8 (D8) or on day 12 (D12) post-ovulation) and treatment order (control and treated cycle; 125 μg of cloprostenol IM on Days 0 through 3). Serum P4 concentrations were measured via ELISA and expressed as area under the curve (AUC) for the period between Days 0 and 8 post-ovulation. For gene expression analysis, target transcripts (PGR, ESR1, ESR2, OXTR, PTGER2, PTGER4, PTGES, PGFS, PTGS1, PTGS2, SCGB1A1, and uterocalin (P19)) were analyzed by quantitative RT-PCR, and data were analyzed with the comparative $\Delta\Delta Ct$ method using B2M as a reference transcript. Changes between control and treated cycles were compared with a paired t-test. Serum P4 concentrations were lower in treated mares (D8: 33.5±1.8 & D12: 19.6±1.0 ng/day/mL^{-1}±SEM) compared to control mares (D8: 98.8±7.2 & D12: 94.7±4.3) ($p<0.0001$). Quantitative RT-PCR demonstrated a down-regulation of PTGES ($p=0.015$) and of P19 ($p=0.08$) in mares with low serum P4 at Day 8. At Day 12, there was an up-regulation of ESR1, ($p=0.001$), PGR ($p=0.003$), SCGB1A1 ($p=0.009$) in treated mares. This study indicates that low serum concentrations of P4 have an effect on the endometrial gene expression during diestrus.

Acknowledgments

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Keywords: Progesterone, gene expression, endometrium, mare

Transcervical endoscopic catheterization technique with uterine lavage to improve clinical outcomes of medically managed pyometra in the bitch

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Pyometra is a breed-related common cause of loss of fertility in the bitch. Many affected bitches become unable to reproduce, either because they are surgically managed by ovariohysterectomy or because of poor response to medical management. Treatment with antibiotics, prostaglandins, and medical supportive care can be augmented by transcervical endoscopic catheterization (TECT) and uterine lavage. The TECT procedure used concurrently with medical management has been hypothesized to lead to improved clinical outcomes leaving the bitch intact and able to be successfully bred again.

The TECT and lavage was performed on five bitches with naturally occurring pyometra presented for medical management. Two were nulliparous, three were multiparous. Since treatment, three bitches have been bred, with two of the three conceiving and successfully carrying litters to term, resulting in live births. The remaining two have not yet been bred as there has not been sufficient time since the procedure for them to return to estrus.

There are brief mentions of using TECT and lavage to improve non-surgical outcomes but the technique and outcomes had limited exposure in publications and application in practice. The TECT technique used will be described.

This technique was similar in equipment and procedures used to breed dogs by endoscopic-guided transcervical insemination (TCI). However, the appearance of the vaginal vault was less crenated in bitches with pyometra during TECT than what is seen during estrus during TCI. Care was taken to avoid damage and perforation of the vagina, cervix, and uterus during the procedure as the tissues were thinner and more friable than during estrus. During the TECT, the bitches were awake and placed in standing position on an elevated table. Fluid was flushed in and out of the uterus via the cervix. The equipment used was either a ureteroscope or cystoscope with or without a TCI shunt system, using a 5 to 8 Fr catheter. The catheter was advanced through the endoscope and through the cervix. During the lavage, ultrasonography was useful to monitor flow of fluid in the uterus. Fluid with or without antibiotics was administered through the intrauterine catheter, to a volume of 100 to 1,000 mL, determined by the patient size and ultrasonic evaluation.

Keywords: Transcervical endoscopic, pyometra, TECT, bitch

A comparative efficacy trial for timethoprim-sulfamethoxazole, gentamicin and penicillin, using an *ex vivo* model of gestational disease

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Treatment of equine placentitis and other infectious reproductive diseases relies heavily on antibiotics. However, clinical efficacy of commonly used antibiotics is poor. In our laboratory, we developed an *ex vivo* model to study antibiotic efficacy under conditions characteristic of pregnancy and uterine disease.

In the current study, we aimed to test antibiotic efficacy of four antibiotics against *Escherichia coli* (EC) and *Streptococcus equi* subsp. *zooepidemicus* (SEZ) in Mueller Hinton broth (MHB), fetal fluids (FF) or purulent uterine fluid (PUF). Known concentrations of bacteria and two physiologically achievable concentrations (high [H]/low [L]) of trimethoprim-sulfamethoxazole (TMS), potassium penicillin (P; SEZ only), gentamicin sulfate (G), or P and G together were added to sterile autoclaved fluid.[1] Each combination was incubated in triplicate for 8 hours, and serial dilutions were plated for quantitative assessment of bacterial load.

In PUF inoculated with EC, antibiotics were not effective, with greater than 3 $\log_{10}$ growth in all samples. Bactericidal activity (3 $\log_{10}$ reduction in bacterial concentration) was achieved by all antibiotics in MHB, and by either TMS or PGH in FF. Bactericidal activity against SEZ was achieved only by PGH, regardless of fluid-type. Penicillin alone (PH, PL) and PGL were bacteriostatic in all fluid types, while TMS and G lost efficacy against SEZ (bacterial growth at 8 hours) in PUF or PUF and FF, respectively.

These findings demonstrate a profound impact of physiologic fluids on antibiotic efficacy, with PG performing best under the study-conditions. Further work to understand the mechanisms by which these antibiotics are inhibited is warranted.

Keywords: Equine, placentitis, antibiotics, *Escherichia coli, Streptococcus equi* subsp *zooepidemicus*

Acknowledgments

Mitsu Suyemoto and Tonya Harris

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Endocrine and molecular changes in the equine follicle associated with ageing in mares

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Reproductive ageing in mares is an important process that is associated with reduced fertility. The objective of this study was to examine age-related differences in follicular dynamics, hormone concentrations, and gene expression in granulosa cells of growing and dominant follicles in mares of various ages. Young (n=10), middle-aged (n=16), and old (n=17) mares were examined using transrectal ultrasonography to track follicular growth during two to three estrous cycles, and total antral follicle counts were determined at least once during each estrous cycle. Granulosa cells were collected from small growing (n=17, 15-20 mm) and dominant follicles (n=14, 35 mm) of excised ovaries after completion of the study. Progesterone and follicle stimulating hormone (FSH) concentrations were measured during the first estrous cycle using an ELISA and RIA, respectively. The gene expression of the FSH receptor (FSHR), luteinizing hormone (LH), LH receptor (LHR), and estrogen receptor β (ERβ) in granulosa cells was examined by qRT-PCR. The influence of age on reproductive parameters and mRNA transcripts was examined using a mixed model. Old mares had a significantly longer inter-ovulatory interval and follicular phase, lower number of antral follicles, and the day of deviation occurred later than in younger mares. The diameter of the pre-ovulatory follicle had a tendency to be smaller in old mares. Concentrations of FSH were significantly higher during the follicular phase in old mares, while progesterone concentrations had a tendency to be higher in old and middle-aged mares. Finally, the expression of FSHR, LHR, and ERβ within growing or dominant follicles was not significantly influenced by mare age. In contrast, the FSHR and ERβ were significantly upregulated in growing follicles compared to dominant follicles whereas the LHR was upregulated in dominant follicles. In conclusion, ageing in mares is associated with reproductive and endocrine changes, whereas molecular changes within the follicle are related to the stage of follicular development, rather than mare age.

Keywords: Age, mare, follicular dynamics, endocrinology, gene expression

Testicular abscess in an Aberdeen Angus bull

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An 18-month old Aberdeen Angus bull presented with semen quality unsuitable for cryopreservation for six months. The left testis was enlarged. The right testis was unremarkable. There was astenozoospermia and teratozoopermia. Ultrasound of the left testicular parenchyma revealed a round 2.5-cm, focal hypoechoic lesion with a hyperechoic center and well-defined margins. Abscess or tumor was suspected. Fine needle aspiration or biopsy were recommended. The owner elected hemicastration and excisional biopsy. Testicular abscess was confirmed on gross examination, impression smears and histopathology; *Staphylococcus simulans* was isolated. Ceftiofur and anti-inflammatories were administered for nine days. Re-evaluation at seven months showed poor semen quality persisted. Anti-sperm antibodies were not present (2.7% and 8.2% IgG- and IgA-bound spermatozoa, respectively). Ultrasound revealed multiple hyperechoic small foci and an abnormal vascular pattern in the remaining testis. Differential diagnosis included orchitis, fibrosis, or calcification. Fine needle aspiration or biopsy was recommended. The owner elected to treat as orchitis with vitamin E and tulathromycin for six weeks. Nine months after hemi-castration, semen quality was still unacceptable.

Semen quality before and after hemicastration

Time	Presentation	7 months	9 months
Sperm motility (%)	0	82	40
Normal spermatozoa (%)	43	5	58
Primary/secondary abnormalities (%)	45/12	85/10	13/29
Round cells (%)	Unavailable	10	0

Causes of testicular abscess include trauma, ascending infection or hematogenous origin.[1,2] Sequelae include contralateral testicular degeneration from thermal injury, expansion of the septic process, release of pro-inflammatory cytokines or autoimmune orchitis. When considering hemicastration, the condition of and potential sequelae to the contralateral testis should be evaluated. Here, damage to the contralateral testis or failure to properly treat pre-existing orchitis may have resulted in continued poor semen quality. While treatment improved semen quality, it was still unacceptable for cryopreservation. The bull was used for natural mating, which was considered ethical since the condition was acquired.

Keywords: Testicular abscess, hemicastration, antisperm antibodies, bull

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Unusual systemic response to artificial insemination in a maiden mare

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An eight year-old maiden Warmblood mare was presented to be bred by cooled-transported semen. Breeding management and insemination occurred without incident. However, 24 hours after insemination, the mare developed a fever, acute onset lameness, and severe distal limb edema. Differential diagnoses for these clinical signs included equine viral arteritis (EVA), equine herpes virus (EHV), influenza, and purpura hemorrhagica.

Because of the contagious nature of these differentials, the mare was immediately moved to isolation. Testing ruled out all differentials including EVA (mare and the stallion tested negative). Within 48 hours, all clinical signs resolved with supportive care, but the mare was not pregnant 14 days later. She was rebred to the same stallion during her subsequent estrus. Her cycle and insemination were without complication. Within 18 hours, the mare again developed a fever, acute onset lameness, and severe distal limb edema. A reaction to the seminal plasma of that stallion or extender used was suspected. Clinical signs resolved within 24 hours with minimal care. The mare became pregnant, so further diagnostics to confirm suspicions were not performed.

Adverse reactions to artificial insemination (AI) are rare in horses,[1] and most are confined to the uterus.[2] This case is unusual in that the clinical signs observed were systemic rather than local (uterus). On both occasions, one stallion was used and semen was shipped in the same semen extender, and both times the observed clinical signs mimicked other disease processes. Further diagnostics were not pursued due to the confirmed pregnancy. Thus an exclusionary diagnosis of a reaction, likely to a component in the semen extender, was suspected due to the repeatability of the clinical signs following insemination and the negative test results obtained. A definitive diagnosis will require infusing the mare's uterus with extender alone, then unextended semen from this stallion, and evaluating each response.

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Metastatic neoplasia in a postpartum Holstein heifer

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Dairy cow postpartum programs identify common health problems; however, they have limitations as to timely diagnosis of uncommon diseases. A 24 month old Holstein heifer delivered a healthy calf on day 274 of gestation. Reproductive evaluation 25 days postpartum demonstrated a non-retractable reproductive tract with cervical and uterine adhesions; at 32 and 46 days postpartum progressive enlargement of the uterus was noted. Ultrasonography showed fluid and fibrin within the abdomen. The heifer developed signs of respiratory disease, fever, decreased milk production, and loss of body condition. No clinical improvement was seen despite anti-inflammatory and antimicrobial therapies. Examination 53 days postpartum confirmed previously noted adhesions and a 30 cm diameter mass associated with the reproductive tract. Ultrasonography demonstrated numerous 2.0-5.0 cm diameter, fluid-filled structures throughout the peritoneal cavity. Due to clinical findings and health deterioration of the animal, euthanasia was elected.

Necropsy findings identified metastatic neoplasia involving the serosa of abdominal viscera and parenchyma of mediastinal and perirenal lymph nodes. Ovaries were not readily identified. Serology for bovine leukosis virus was negative. Microscopically, masses consisted of round neoplastic cells in dense sheets, spindle-shaped cells, and multinucleate giant cells, which were positive for vimentin but negative for cytokeratin, lysozyme, and CD68 via immunohistochemistry. Findings were consistent with disseminated histiocytic sarcoma.

This case is relevant to theriogenologists as it highlights the importance of postpartum evaluation programs to identify and address pathologies. However, animals which do not respond to treatment protocols for common diseases should be further evaluated. In this case, the heifer was treated for infectious respiratory disease when a mediastinal neoplastic mass was compressing the lungs. Only identification of reproductive tract abnormalities indicated a need for further evaluation. Finally, this case raises questions about the interrelationship between pregnancy and development or rate of progression of neoplasia, a topic which warrants further investigation.

Keywords: Bovine, pregnancy, ultrasonography, sarcoma, immunohistochemistry

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