Laboratory use in pregnancy loss diagnosis

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Abstract

Pregnancy loss and perinatal mortality are important economic and public health issues for the livestock industry. To minimize losses and prevent human infections, rapid and accurate identification of the cause is critical. Veterinary diagnostic laboratories not only have an important role in identifying causes of pregnancy loss but also in monitoring (surveillance) infectious diseases that cause reproductive failure. Appropriate specimen collection and handling are essential prerequisites for reliable test results. Consequently, laboratory test procedures and their value may be compromised by using specimens that are not collected, labelled, handled or stored properly prior to testing. Principles of biosecurity and biocontainment must be strictly followed during sample collection. For accurate determination of the cause of pregnancy loss in ruminants, it is recommended to use a battery of diagnostic tests done in parallel. These tests include histopathology, real time polymerase chain reaction (rtPCR) assay, bacterial and viral isolation, detection of fetal antibodies in serum or fluid from thorax or abdomen, and maternal serology. Veterinary laboratory professional staff can provide consultation services to clients regarding test selection and testing strategies, interpretation of results, surveillance, disease prevention and control programs. This review provides essential information on collection of diagnostic samples and interpretation of test results for the diagnosis of most common infectious causes of pregnancy loss in ruminants.

Keywords: Ruminants, abortion, diagnosis, pregnancy loss, laboratory

Introduction

Pregnancy loss is an important cause of economic loss to livestock producers. Additionally, many infectious causes of pregnancy loss in ruminants are zoonotic agents posing a health risk for livestock owners, animal handlers, and veterinarians.[1-3] The investigation goal in any animal disease outbreak is to identify the cause rapidly and accurately. Determining the cause of pregnancy loss or neonatal death in ruminants is often unsuccessful.[4] Aborted fetuses often die in utero and are autolyzed when received at the laboratory, hampering interpretation of postmortem findings.[5] Consequently, a specific cause was identified in only ~ 30% of midterm and late-term pregnancy losses in cattle and 57% in sheep.[1,2,6] However, this should not be considered a diagnostic failure. In a majority of cases, the diagnostic emphasis is towards ruling out infectious causes of abortion. When results are negative, noninfectious causes of abortion may be suspected; however, infectious causes cannot be completely ruled out. Veterinary diagnostic laboratories have an important role in the identification of causes of pregnancy loss and in surveillance of infectious diseases that cause reproductive failure. Additionally, they also support interstate and international livestock trade by providing testing to ensure compliance with the conditions and measures necessary to minimize potential risks associated with animals and animal products.[7,8]

Appropriate specimen collection and handling are essential prerequisites for accurate test results. The value of a laboratory test procedure may be compromised by using specimens that are not collected, labelled, handled or stored properly.[8] This requires a combined effort among various people (owner, animal caretaker, and veterinarian) and the veterinary diagnostic laboratory. In this process, the owner and the animal caretaker need to provide an adequate clinical history that includes clinical signs, nutrition, breeding program, history of vaccination, time and type of vaccines, medications, and other pertinent information. Based on the information provided, the veterinarian has to establish a preliminary diagnosis and obtain adequate samples for the diagnostic laboratory.[9] Samples collected without clear directions often do not yield meaningful results.

Ruling out all possible causes of pregnancy loss may be costly. Most diagnostic laboratories prioritize tests for the most common causes of infectious abortion and those with zoonotic potential.[4] The diagnostic samples should preferably be submitted using an overnight delivery service. Most diagnostic laboratories do not accept routine shipments on weekends or holidays. All veterinary laboratory diagnostic tests must be validated following the guidelines of the American Association of Veterinary Laboratory Diagnosticians requirements for an Accredited Veterinary Medical Diagnostic Laboratory and the World Organization for Animal Health.[10]

Principles of biosecurity and biocontainment must be strictly followed during sample collection. Biosecurity refers to programs for infectious disease control that reduce/prevent the introduction of new diseases into an operation from outside sources. Biocontainment refers to programs that reduce/prevent the movement of infectious diseases within the operation or animal groups.[11] This review provides essential information on the collection of diagnostic samples and the interpretation of test results for the diagnosis of most common infectious causes of pregnancy loss in ruminants. A more detailed description of the cause, pathogenesis and risk of human infection of these diseases is reviewed in the proceedings.[1-3]

Collection and submission of laboratory samples

Procedures for submission of diagnostic samples to the veterinary diagnostic laboratory are complex and must follow multiple regulatory shipping requirements enforced by multiple entities.[12] Along with laboratory samples, the veterinarian should submit a detailed clinical history that includes owner's name, animal species, breed, sex, age, animal identification, nutritional program, clinical signs, percent animals aborting, stage of pregnancy when abortion occurred, details of any fetal malformation, treatments and vaccinations, and environmental influences. Diseases suspected and tests requested should be clearly stated in the submission form.[9] Providing more information to the laboratory usually results in faster and more accurate test results. Laboratory professional staff can provide consultation services to clients in areas of test selection and testing strategies, interpretation of results, and disease prevention, surveillance, and control programs. Many diagnostic laboratories now offer cattle and small ruminant abortion diagnostic plans. Tests included in diagnostic panels vary among laboratories and animal species but in general include histopathology, bacteriology, serology, real time polymerase chain reaction (rtPCR) assay, the latter for bovine viral diarrhea virus (BVDV)/Border disease virus (BDV), bovine herpes virus-1 (BHV-1), *Leptospira* spp., *Chlamydia abortus*, *Coxiella burnetii*, *Neospora caninum*, *Toxoplasma gondii*, in addition to determining vitamin A levels and performing a trace mineral panel.[13,14] The success of determining the cause of abortion in ruminants largely depends on the type and quality of samples submitted to the diagnostic laboratory. Submission of fetal membranes increases the probability of making a diagnosis by 2.3 times.[15]

Whenever handling biological material, from either live or dead animals, the risk of zoonotic disease should be considered and precautions taken to avoid human infection.[16] It is strongly recommended that debilitated older individuals, pregnant women, immunocompromised people and children are not involved in collecting samples and visiting or cleaning birthing areas. Proper personnel protective equipment including coveralls, disposable rubber gloves, rubber boots, safety goggles or masks, and when possible N95 masks or 'powered air purifying respirators' should be worn when handling samples. When a zoonotic disease is suspected, this should be clearly indicated on the submission form. In small ruminants, all abortions should be considered potentially zoonotic, and principles of biosecurity and biocontainment strictly followed.[17,18]

Samples must be packaged properly to prevent leakage of blood or tissue fluids during shipment and ensuring that they arrive at the laboratory in good condition. Shipments must comply with all applicable laws (local, state, and federal) governing packing, marking, and labeling.[12] Submission of most laboratory samples requires a watertight inner primary container, a watertight inner secondary container and a sturdy outer shipping box (tertiary container). Inclusion of absorbent materials inside the secondary container is recommended to minimize contamination of other packages in case a container breaks or leaks. Tissue samples for histopathology should be submitted in 10% buffered formalin. If formalin is not available, tissue samples must be sent chilled with cold packs but not frozen.

Collection and submission of laboratory samples from aborted fetuses

In abortion cases, particularly in small ruminants, it is recommended to submit the entire fetus and fetal membranes to the diagnostic laboratory to prevent exposure of personnel to zoonotic agents and to avoid contamination of the premises. Fetal membranes are very important to identify the cause of abortion and when available should be submitted along with the fetus. Examination of fetal membranes can yield information that may be important in the selection of ancillary tests and interpretation of laboratory results. Fetal membrane lesions of diagnostic significance can be recognized for many economically important infectious abortifacient diseases.[19] Poor viability of cloned bovine fetuses during days 35 - 60 has been associated with poor chorioallantoic development.[20] Concurrently, along with fetal membranes and fetus, submission of serum samples from animals that have aborted and from those that had normal deliveries in the same herd/flock is recommended.

When submission of the entire fetus is not possible, a field necropsy should be performed, and complete sets of tissues chilled on ice or fixed in 10% buffered formalin submitted to the laboratory. Necropsies should be performed as soon as possible after abortion. Necropsies of aborted fetuses and examination of fetal membranes can provide a wealth of information that can help to establish animal health prevention and control programs.[21]

Safety during field necropsies is of paramount importance to avoid exposure of personnel and contamination of premises. Personnel protective equipment should be used during necropsy procedure. At the end of necropsy, all remaining tissue should be properly disposed, and instruments and equipment should be thoroughly disinfected. Fetus and fetal membranes should be shipped to the laboratory, as indicated above. Adequate cooling packages should be included within

the secondary container but should not come in direct contact with samples.[11]

Preparation of tissue samples for histopathology

Tissues for histopathology should be collected as soon as possible during necropsy, but not frozen to avoid autolysis. With the exception of brain, these tissues should be < 1 cm thick (to promote fixation) but can be 3 - 5 cm in length and width. They should be submitted in 10% phosphate buffered formalin (10 volumes of formalin per volume of tissue) in plastic jars with properly sealing lids.[8,22] Tissues for histopathology should include at least samples from 3 cotyledons and 3 sections of intercotyledonary area, brain (entire half of the brain including brain stem and cerebellum), heart, lung, thymus, liver, spleen, kidney, adrenal gland, and skeletal muscle. In general, tissues from the gastrointestinal tract of aborted fetuses are of limited diagnostic value, although they are important in cases of neonatal death. A second set of tissues and stomach contents should be collected, chilled and sent in individual Whirl-Pak or Ziploc bags for microbiology, molecular analyses, virology and/or toxicology. Fresh tissues should be larger than fixed tissues and be ~ 3 - 6 cm thick.[8].

Preparation of tissue samples for bacteriology and mycology

Fetal tissues and fluids for culture should be aseptically collected individually in sealable Whirl-Pak or Ziploc bags or sterile tubes and sent chilled with cold packs. Saline, broth or formalin should not be added to the samples.[22] Preferred samples for microbiology diagnosis in abortion cases include fetal membranes, fetal stomach content, fetal tissues including lung, liver, and brain.

Preparation of tissue samples for molecular analyses

In the last 2 decades, real time rtPCR has been the pillar in the diagnosis of infectious abortion in ruminants. Tissue samples for detection of microbial nucleic acid by PCR can be submitted refrigerated in individual Whirl-Pak or Ziploc bags or sterile tubes chilled with cold packs. Blood should be collected and thoroughly mixed in EDTA tubes (purple tops) and not in heparin tubes (green tops). Clotted blood is not suitable for molecular testing. Bacterial culture swabs submitted in bacterial culture media (gel, charcoal) are not appropriate for rtPCR testing. Swabs for rtPCR testing should be moistened with 1 - 2 drops of sterile saline and submitted in sterile leak-proof containers (e.g. red top tubes).[14]

Amount of nucleic acid (DNA or RNA) of specific pathogens present in a given sample is determined in rtPCR assays that amplify specific sections of microbial nucleic acid using fluorescent-labelled oligonucleotide probes, with results given as cycle threshold (Ct) values. The cutoff value provides classification of results as either 'detected' or 'not detected' and may also include an indeterminate range. Using fluorescent probes, these assays monitor the increase in target nucleic acid as it is amplified over a total of 40 - 45 rtPCR cycles. The cycle number at which this threshold is crossed is termed the 'cycle threshold' or 'Ct.' The fewer the cycles needed to reach the Ct, the more nucleic acid of that specific microbial agent is in the sample. For example, Ct values < 28.0, 28.1 - 35.0, and 35.1 to 40 - 45.0 indicate abundant, moderate or minimal amounts, respectively, of target nucleic acid in the sample. In veterinary diagnostic laboratories, dilution series of the target nucleic acid are not included. Generally, only a single positive amplification control concentration is routinely included for rtPCR assays, thereby limiting the precision of quantification and making most of the assays semiquantitative rather than absolute. Furthermore, rtPCR assays should be validated and incorporate either an exogenous or endogenous internal control.[23,24]

Preparation of tissue samples for analytical chemistry

Samples of fetal tissue, in particular liver and kidney, should be placed in sealable containers and chilled. When vitamins or mineral deficiencies are suspected, 10 grams of fetal liver should be placed in additive-free transport containers and chilled with cold packs. For vitamin A determination, samples should be protected from light immediately after collection and sent to the laboratory on ice by an overnight courier. Since abortions can be the result of nutritional deficiencies or toxins, it is recommended to submit both feed and water for analysis. Maternal blood collected in purple and red top-top tubes (EDTA and serum; both samples are required) can be submitted for metal and mineral panel determination by inductively-couple plasma/mass spectrometry.[25]

Preparation of samples for serology

Fetal fluids, fetal blood (from the heart) and maternal blood for serology should be collected in sterile red-top glass tubes or separator tubes and placed into padded, protective containers for shipping. Placing glass blood/serum tubes between frozen ice packs without protective padding will often result in tubes breaking and leaking.

Interpretation of maternal serology testing for the diagnosis of infectious causes of abortion can be difficult. Use of threshold serum titers or paired serum samples collected at abortion and 2 - 3 weeks later is a common practice. However, these practices may not be accurate with some infectious agents such as BVDV, infectious bovine rhinotracheitis, neosporosis, and leptospirosis, due to the time lag between infection and abortion. Lack of change in serum antibody titer in acute and convalescent samples has been reported for leptospirosis, BVDV, and infectious bovine rhinotracheitis. Interpretation of results is further complicated in animals that have been vaccinated, since serologic tests do not differentiate between vaccine- and infection-induced antibodies. An alternative diagnostic approach suggested is the collection of blood samples from groups of

animals that have aborted and from herd mates that maintain pregnancy or had normal parturition.[26]

A 2-by-2 table (see below) is used to classify pregnancy loss and nonpregnancy loss females according to their exposure to the risk factor being evaluated. The degree of association between the risk factor and pregnancy loss is measured by calculating the odds ratio (OR), i.e. the ratio of the odds of pregnancy loss for females exposed to the risk factor to the odds of pregnancy loss for females not exposed to the risk factor. In the case of antibodies, titers are necessary to establish a threshold level of risk factor present or absent.

An OR of 1 implies that there is no association between the risk factor and pregnancy loss. An OR > 1 implies that the risk factor is associated with an increased risk of pregnancy loss. An OR < 1 suggest that females with the risk factor have a decreased risk of pregnancy loss. In general, a risk factor with an OR of > 3 may be considered significant and they may act to eliminate or alter that risk factor. This odd ratio depends on an adequate number of samples and the use of correct statistical analysis (Fisher's Exact test or Chi-square analysis) to arrive at a sound conclusion. An OR calculated using a single 2-by-2 table may not be accurate in herd situations in which more than 1 risk factor directly influences the same production parameter.[27]

	Pregnancy loss	Nonpregnancy loss
Risk factor present (+)	Pregnancy loss with risk factor (A)	Nonpregnancy loss with risk factor (B)
Risk factor absent (-)	Pregnancy loss without risk factor (C)	Nonpregnancy loss without risk factor (D)

The OR is calculated from the 2-by-2 table using the following equation: OR = A X D / B X C

To increase the diagnostic value of the maternal serum agglutination test (SAT) for the diagnosis of *Salmonella dublin* culture-positive bovine abortion and stillbirth, the predicted probability values, rather than the traditional arbitrary breakpoints of negative, inconclusive and positive, increased the diagnostic value of the maternal SAT. Information previously considered inconclusive can be derived from test results by using predicted probability values.[28]

Placentitis

Placenta is the organ most frequently affected in cases of infectious abortion in ruminants. The etiological agent is often identified during histologic examination of fetal membranes or can be detected with ancillary tests. Many bacteria and fungi that cause abortion in ruminants cause placentitis including brucellosis, campylobacteriosis, chlamidiosis, coxiellosis, listeriosis, yersinosis, and aspergillosis. Apicomplexan parasites such as *Neospora caninum* and *Toxoplasma gondii* also cause placentitis and the organisms can be associated with fetal membrane lesions in some cases.[29] Inflammation of fetal membranes is often not uniform. Entire fetal membranes can be submitted double-bagged and chilled on ice, or multiple portions of cotyledonary and intercotyledonary areas, fixed in 10% formalin for histopathology and chilled on ice for bacterial/fungal culture and molecular analysis, should be submitted.

Fetal malformation

Causes of congenital fetal malformations can be genetic, toxic, nutritional, and infectious. In cattle, BVDV fetal infection can cause fetal malformation.[30,31] Infection with BDV in ruminants during the second or last trimester of pregnancy causes fetal malformation, fetal mummification, abortion, stillbirth, and birth of weak or normal lambs.[32]

In North America, Cache Valley virus (CVV) is a common cause of fetal malformation in small ruminants and less frequently cattle.[33,34] Experimental infection of pregnant sheep with 2 California serogroup bunyaviruses, LaCrosse virus and San Angelo virus, and a Bunyamwera serogroup member, Main Drain virus, induced a range of lesions similar to those induced by CVV including arthrogryposis, hydrocephalus, fetal death, axial skeletal deviations, anasarca, and oligohydramnios.[35] Fetal teratogenesis in sheep, goats and cattle have been described in natural and experimental infections with exotic Bunyaviruses including Akabane virus, Schmallenberg virus, Rift Valley fever virus, and Wesselsbron disease virus.[36,37]

Vertical transmission of bluetongue virus (BTV) from dam to fetus in enzootic areas is considered negligible. In these areas (including the US), BTV-induced pregnancy loss and congenital malformations have been associated with BTV strains from modified live virus vaccine strains.[38] Infection of cattle, sheep and goats with the European BTV-8 wild-type strain demonstrated a high incidence of transplacental transmission and pregnancy loss in natural circumstances.[39]

Veratrum californicum in sheep, *Astragalus pubentissimus* (locoweed) and benzimidazoles are toxic causes of fetal malformation in sheep and cattle.[40-42] Genetic causes of fetal malformation include abnormalities of the chromosomes or genes. Bovine

fetal abnormalities have been reviewed in detail.[43]

Fetal mummification

In cattle, fetal mummification occurs after formation of placenta and fetal ossification. Fetal mummification must be differentiated from fetal maceration, in which the fetus putrefies due to bacteria and oxygen entering the uterus when the cervix is open.[44] Mummification of bovine fetuses is uncommon and cows do not always respond to treatment with prostaglandin $F_{2\alpha}$. Most often, mummification occurs between 3rd and 8th months of pregnancy without lysis of the corpus luteum and the cervix remaining closed.[45]

Mummification of a fetus occurs when it dies in utero and undergoes subsequent autolysis in the absence of bacterial infection and putrefaction. A definitive etiology is rarely determined, due to tissue degeneration and autolysis. Mummification process usually renders worthless the analysis of bacteria, protozoa and viruses by standard methods, but nucleic acid of some abortifacient infectious organisms can be detected by rtPCR in fetal tissues. Proposed causes of fetal mummification in cattle include bovine viral diarrhea, leptospirosis, and neosporosis.[45] In a study, 25% of bovine mummified fetuses were positive for *Neospora caninum* DNA by PCR.[46] In small ruminants, fetal mummification is often the result of infection with Border disease virus, *Chlamydia abortu*s, *Coxiella burnetii*, or *Toxoplasma gondii*.[44]

Chlamydiosis

Chlamydiosis caused for *Chlamydia abortus* is an important cause of late-term abortion in small ruminants. The complement fixation test is used by many veterinary diagnostic laboratories to detect Ch. abortus antibodies in maternal serum. Complement fixation titers of 1:16 - 1:32 are considered positive for most laboratories. The titer may increase to 1:80 or higher 2 - 3 weeks after abortion.[47] The complement fixations test is not *Ch. abortus* specific and positive titers can arise from cross reactivity to *Ch. pecorum*. Several enzyme-linked immunosorbent assay (ELISA) tests with varied specificity and sensitivity have been used for the diagnosis of chlamydiosis.[48]

A fluorescent antibody test is used by some veterinary diagnostic laboratories to detect *Ch. abortus* in fetal membranes, liver or spleen. An rtPCR assay is used for the detection of *Chlamydia* spp nucleic acid, but this test detects *Ch. psittaci*, *Ch. felis* and *Ch. abortus* and does not distinguish among them.[13,14]

Coxiellosis

Coxiellosis, caused by the intracellular bacterium *Coxiella burnetii*, is an important zoonotic disease that causes pregnancy loss in small ruminants and less commonly in cattle and other species. In the US, *C. burnetti* is a class B bioterrorism select agent subject to the select agent external icon regulations (42 CFR Part 73 of the Electronic Code of Federal Regulations). In some veterinary diagnostic laboratories, in all cases of small ruminant abortions, the standard operating procedure is to first rule out *C. burnetii* by rtPCR before proceeding with other diagnostic tests.14 When a case is positive for *C burnetii*, all tissues and samples are appropriately disposed and no further testing is done.

Detection of *C. burnet*ii antibodies in maternal serum is by ELISA, immunofluorescence assay or complement fixation. Antibodies in maternal serum indicate exposure to *C. burnetii* but do not confirm *C. burnetii* as the cause of abortion. However, lack of antibodies in maternal serum rules out coxiellosis as the cause of abortion.[16] *C. burnetii* IgM and IgG phase II specific antibodies in adult animals can be detected 2 weeks post-infection and remain increased for up to 13 weeks. Antibodies directed against *C. burnetii* phase I increase generally 4 weeks later compared to the phase II antibodies. Serum antibodies in infected animals can be detected for several months up to years. All rtPCR positive results in aborted fetuses should be corroborated by testing maternal serum for *C. burnetii* antibodies.[19,49]

Leptospirosis

Leptospirosis is an important zoonotic disease caused by > 260 antigenically distinct serovars belonging to 25 serogroups grouped in 9 pathogenic species, 5 intermediate and 6 saprophytic species of *Leptospira*, a gram-negative bacterium belonging to the order Spirochetales.50 Leptospirosis can cause abortion, fetal mummification, stillbirths, the birth of weak calves, and infertility in cattle. Several species of *Leptospira* cause abortion in cattle. *Leptospira* species can be classified as host-adapted, infecting maintenance host animals, or nonhost-adapted, infecting accidental host animals. Most common leptospira species causing bovine abortions and infertility in North America is *Leptospira borgpetersenii* serovar *hardjo*.[51] Cattle that become chronically infected with serovar hardjo serve as reservoirs for infection to other cattle and humans. Some cattle with chronic hardjo infections have low or no agglutinating antibody titers.[52,53]

Diagnostic tests for leptospirosis include detection of serum antibodies and demonstration of the organism or its DNA in tissues or body fluids of aborted fetuses by rtPCR or other tests. Due to its low cost and wide availability, a microscopic agglutination test (MAT) is the most common method used for demonstration of antibodies in animal serum. With MAT however, determining the stage of infection can be difficult in cases of bovine abortion or stillbirth, as infection usually occurs 1 - 4 weeks before the expulsion of the fetus and by this time, MAT titers would have stabilized. Some consider the presence of high serum antibody titers satisfactory to establish a diagnosis. In some serovars such as *Pomona*, *Grippotyphosa*, *Icterohaemorrhagiae* and *Canicola*, maternal serum antibody titers > 1,600 appear to correlate with abortion. However, maternal antibodies in abortion caused by serovar hardjo are often lower or negative at abortion; therefore, a low antibody titer does not rule out leptospirosis. Antibodies to *Leptospira*

spp. can be demonstrated in serum of some aborted fetuses at dilutions of 1:10, but some fetuses can be positive by rtPCR without antibodies in pleural fluid.[54]

The MAT does not differentiate between vaccine-associated antibodies and those from an active infection. In general, vaccinated animals develop low agglutination titers that last 1 - 3 months. However, some outliers develop high antibody titers that persists for several months after vaccination. Additionally, cross reaction between serovars occurs with MAT, complicating interpretation of results.[52] New methods for the detection of *Leptospira* serum antibodies have been developed but are not widely used by veterinary diagnostic laboratories. The microsphere immunoassay (MIA), which uses Luminex xMAP technology, had better sensitivity compared to MAT and it determined reactive from nonreactive antibodies and differentiated IgM from IgG responses.[55]

Currently, the most common test for the diagnosis of *Leptospira* abortion in cattle is rtPCR done on tissues of aborted fetuses. The preferred fetal tissue is kidney, although other fetal tissues can be used. Veterinary diagnostic laboratory rtPCR assays will amplify *Leptospira* DNA of all serotypes, but will not differentiate between serotypes. Other tests used for the diagnosis of leptospirosis in aborted fetuses are immunofluorescence for demonstration of the organism in urine and tissues and immunohistochemistry or special stains for demonstration of the organism in formalin-fixed, paraffin-embedded tissue sections. However, these methods are considered to have low sensitivity and are infrequently used.

Cache Valley virus

Cache Valley virus is an arbovirus of the family Bunyaviridae that is endemic in North America and infects a wide range of domestic and wild animals, and humans. Viral infection is usually asymptomatic in adult sheep, goats, and cattle. Viral infection during pregnancy may result in embryonic mortality, fetal mummification, fetal malformation, and pregnancy loss in sheep and goats, and less often in cattle.[33,34]

During natural CVV infection of pregnant ruminants, CVV may cross the placenta, infect the fetus and cause congenital arthrogryposis and hydranencephaly.[56] The congenital malformations induced by CVV during spontaneous infections in sheep have been reproduced experimentally by intrauterine inoculation of the virus between days 27 - 54 of pregnancy.[57] Experimentally infected ovine fetuses mount an innate and adaptive immune response that presumably contributes to viral clearance in infected animals.[58] Virus isolation from full-term aborted fetuses with CVV-induced malformations is unsuccessful. Some diagnostic laboratories offer a gel-based PCR for the demonstration of CVV-RNA in tissues of aborted fetuses. However, as cross contamination in gel-based PCR platforms is common, their use is discouraged. An rtPCR assay has been developed for the detection of California serogroup viruses and CVV, for use in human surveillance, but is not routinely used for CVV in small ruminants.[59] Until rtPCR methodology is validated in small ruminants for detection of CVV-RNA in late-term malformed ruminant fetuses, the results of gel-based PCR are difficult to interpret.

Determination of CVV neutralizing antibodies in fetal serum from blood clots in the heart or in fetal fluids is the most reliable test to diagnose CVV fetal infection in late-term malformed fetuses. The presence of CVV antibodies in maternal serum is an indication of exposure but not proof that fetal malformation was caused by CVV. The lack of antibodies in maternal serum rules out CVV as the cause of fetal malformation. The presence of serum CVV antibodies before breeding in ewes and goats is protective of transplacental fetal infection with CVV but not against other related bunyaviruses.[33,34]

Bluetongue

Bluetongue is an insect-transmitted, noncontagious disease of ruminants caused by bluetongue virus (BTV), the prototype virus of the genus Orbivirus in the family Reoviridae. In endemic areas, abortion, stillbirth, and fetal deformities have been attributed to tissue culture adapted vaccine strains strains of BTV in sheep and cattle, and less often goats.[38] During the 2006 European BTV outbreak, the ability of BTV-8 field strain to cross the placenta and infect the fetus was a major concern because of transplacental transmission and increased abortions, stillbirths, and fetal deformities in cattle.[60] Severe brain lesions were observed in the brain of bovine fetuses 12 or 20 days after BTV-11 experimental inoculation at 120 days of pregnancy. Virus was recovered from brain, blood and a pool of lung and liver from all experimentally infected animals. Viral group-specific antibodies were detected in 2 of 8 inoculated fetuses by the agar gel immunodiffusion test.[61]

Serological assays available for demonstration of BTV antibodies include complement fixation, virus neutralization, agar gel immunodiffusion test, and several ELISA formats. Virus isolation is done by inoculation of susceptible sheep or embryonated chicken eggs with heparinized blood or homogenized lymph nodes, spleen, or lung of infected animals. Blood and tissue samples for diagnostics need to be preserved at 4˚C, but not frozen. The rtPCR assay is a commonly used diagnostic test with high sensitivity and specificity. A positive rtPCR result does not necessarily indicate the presence of infectious virus, as it can detect BTV nucleic acids after the virus is no longer viable. Antibodies can be demonstrated in precolostral serum samples of lambs infected in utero and the virus also can be isolated in some of these lambs due to immune tolerance and persistent infection.[62]

Bovine viral diarrhea

Bovine viral diarrhea (BVD) is an important infectious disease in most cattle-producing countries worldwide and is caused

by bovine viral diarrhea virus (BVDV), a member of the genus Pestivirus in the Flaviviridae family.[63] Diagnosis of BVDV infection is challenging, due to numerous virus types and subtypes and the variety of clinical syndromes induced by BVDV, including animals that are persistently infected (PIs).[64] Currently, phylogenetic analysis has identified [21] Pestivirus subtypes (BVDV 1a-u) and 4 Pestivirus subtypes 2 (BVDV 2a-2d). Four Pestivirus H subtypes (HoBi a-d) have been identified in Europe. Although not currently reported in the US, Pestivirus H subtypes are a concern since they are not routinely detected by current diagnostic tests, and current vaccines may not fully protect against these subtypes.[65] Although of minor clinical importance, infections of cattle with BDV, a small ruminant Pestivirus, can interfere with BVDV eradication programs.[66] Current laboratory techniques for BVDV testing in cattle samples have not been critically validated for their use to test for BVDV in heterologous species.[64]

Virus neutralization tests for BVDV 1a, 1b, and 2 are used for demonstration of BVDV antibodies in animal serum. Interpretation of the results should be in conjunction with vaccination history, including type of vaccine and interval from vaccination to sample collection. Presence of neutralizing antibodies in nonvaccinated calves 6 - 12 months of age is highly correlated with virus circulation in the herd. After administration of killed or modified-live vaccines, titers range from 32 - 256 and from 512 - 1536, respectively, several months after vaccination. However, PI animals generally have no or low antibody titers. BVDV naïve animals that become infected generally have titers in the low to mid thousands, but will decrease to levels similar to those observed with MLV vaccines. Determining acute and convalescent serum samples may provide definitive proof of recent infection.[13]

Acute infection can be detected by virus isolation or rtPCR from serum, blood, nasal swabs, tracheal wash, semen or from lung or lymphoid tissue recovered at necropsy from adult animals and aborted fetuses. Blood for rtPCR should be collected in EDTA tubes. Clotted blood or blood collected in green tops are not suitable for rtPCR testing. BVDV persistent infection can be diagnosed on paired samples taken 3 weeks apart by virus isolation, antigen capture ELISA and rtPCR in serum, whole blood, milk or skin or by immunohistochemistry on ear notch biopsies. Up to 24 ear notches can be pooled and tested by rtPCR initially. When a pool of ear notches gives a positive result, individual samples are tested for identification of individual infected animals.[14] Control and eradication of BVDV infection in cattle herds involves a multidimensional approach that includes the identification and culling of persistently infected animals, vaccination, and biosecurity.[64]

Sentinel serology

A critical step in the control and eradication of BVD is identification and elimination of PI calves. Determining the BVDV infection status in large herds by testing every animal may be labor intensive and costly. A system that has been used to overcome this problem consists of sentinel serology. In this approach, usually a small group of seronegative, nonvaccinated calves are introduced in the herd and subsequently tested for the presence of BVDV neutralizing antibodies. Calves used as sentinels have to be ~ 6 - 12 months old with no BVDV colostrum antibodies that could interfere with serological testing and also that are not vaccinated against BVDV.[67] If there has been no vaccination (sentinel calf program), then both tests (BVDV-1 and BVDV-2) should be requested, as a low value to 1 type might show a 10-fold higher unequivocal titer to the other type. In this instance, the higher titer value would be indicative of an infection with that type of virus.

Neosporosis

Neosporosis in cattle is usually manifested in 2 patterns that may overlap. In the endemic pattern, there is an increased abortion rate in the herd, generally > 5% per year. This pattern persists year after year over a long period. In the epidemic pattern, > 30% pregnant cows may abort with a few months.

Midterm abortion is the characteristic of neosporosis in cattle with no other clinical signs. Not all seropositive cows abort. Congenital neosporosis occurs in 80 - 90% of calves born to seropositive cows. In many cases, congenital infection results in the birth of persistently infected calves that have precolostral antibodies against Neospora. These calves can transmit the infection to their offspring when they become pregnant and perpetuate the infection in the herd. Less often, congenitally infected calves have neurological deficits at birth ranging from mild proprioceptive deficits to severe paralysis, resulting from inflammatory lesions in the brain and spinal cord.

Currently, the main diagnostic test to identify Neospora in tissues from aborted fetuses consists of detection of *N. caninum* DNA by rtPCR. Fetal tissues most often used for rtPCR are brain, heart, lung, liver, and fetal membranes. Demonstration of microscopic lesions in H&E-stained sections of brain, heart, liver, skeletal muscle and fetal membrane, and demonstration of the organism in tissues by immunohistochemistry are recommended as complementary tests. *Neospora* antibodies in fetal serum or fluids can be demonstrated in some aborted fetuses by indirect immunofluorescent antibody test.

Presence of *Neospora* antibodies in maternal serum can be assessed by indirect immunofluorescent antibody test or by ELISA. These tests are not always standardized across various diagnostic laboratories and appropriate cut-off values should be established using standardized sera. Neospora serologic tests in maternal serum should be interpreted with caution and are useful for determination of herd exposure but not to identify Neospora as the cause of abortion in individual cases. Antibody titers in maternal serum may fluctuate throughout pregnancy and in some cases, titers may be below the cut off value at the time of abortion. Regardless of aborting cows' titers below the cut

off value, it is appropriate to compare the presence of Neospora antibody titers between groups of cows that aborted to groups of cows that did not. The percentage of abortion in seropositive pregnant cows is 3 times higher than in seronegative cows and proportion of repetitive abortion in subsequent pregnancies was up to 5 times higher in seropositive cows than in seronegative cows.68 In goats, there was a significant association between increased maternal IgG titers in serum in the second half of pregnancy and congenital transmission of *N. caninum*.[26,69,70]

Conclusion

There are few perfect diagnostic tests, as it is usually possible to have false-positives and false-negative test results. Therefore, it is impossible to prove that a herd/flock is free from a disease even with large sample size, as there is a chance that an infected animal may have been missed or that the test result is wrong.[71] For accurate determination of the cause of abortion in ruminants it is important to take a multidimensional approach and use a battery of diagnostic tests done in parallel, including histopathology, rtPCR, bacterial culture, virus isolation, fetal antibodies in serum or fluid from thorax or abdomen, and maternal serology.[72] Ultimately, linking molecular, microbiological and serological findings to grossly and/or microscopically visible lesions is always recommended to mitigate against false-positive and false-negative results.[4]

Conflict of interest

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