Canine placental microbiome

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Abstract

Placenta is a pregnancy-specific organ responsible for maintenance of gestation and offspring survival in utero. Similar to other immune-privileged sites, fetoplacental unit was considered sterile. However, there is now strong evidence in humans that placenta harbors a unique microbiome. Although this work is in its infancy in veterinary medicine, there are accumulating metagenetic data to support the role of maternal reproductive tract microbiome in pregnancy success and adverse outcomes via placenta. This paper will review and highlight the emerging field of metagenetics in canine reproductive medicine. It will summarize what is known in humans, as well as veterinary species, with an emphasis on future metagenetic characterization of a canine placental microbiome and its potential role in pregnancy outcomes.

Keywords: Dog, placenta, uterus, vagina, microbiome

Introduction

Placentation

Mammalian placenta is a unique and transient organ of pregnancy composed of fetal membranes in intimate association with the mother's uterus. In dog, embryo first enters uterus approximately 11 days after luteinizing hormone (LH) surge.[1] Embryos then traverse the uterine body and horns for several days before attaching to endometrium at approximately 22 days after LH surge and the trophoblast invasion process begins to establish an uteroplacental interface and subsequently a fetoplacental unit. Resulting canine placenta is grossly defined as having a zonary shape, due to an interdigitating zone of contact between endometrium and chorionic sac.[2] Microscopically, this contact has a degree of intimacy that places trophoblast cells in direct proximity to the interstitium of endometrium, but not endometrial blood vessels.[2] Therefore dogs have an endotheliochorial type of placentation, which is different to hemochorial type in humans that have trophoblast cells exposed to maternal blood. Horse placenta is diffusely villous and an epitheliochorial type,[2] which has 6 cell layers between maternal and fetal blood, giving it even less intimacy than dog. These macroscopic and microscopic differences in placentation are important to keep in mind when making translations between human microbiome data and that of veterinary species, including dogs.

Metagenetics, metagenomics, and microbiome

Due to the emergence of this exciting and novel field in veterinary medicine, academicians and clinicians alike are rapidly learning microbiome terms and concepts. Entire microbiota community is considered when assessing the microbiome of any given environment. Microbiota consists of resident bacteria, archaea, fungi, protists, and viruses in the environment of interest. Microbiome is therefore the collective genomes of the microbiota community members and the study of this has been termed metagenomics. Study of an environment's metagenome, where scientists utilize the principles of molecular biology and genetics, is referred to as metagenetics. Sequencing of metagenome results in read lengths of significant depth that bacterial genus and species can be accurately identified and organized into operational taxonomic units (OTUs) that are mapped to previously generated bacterial taxonomic databases. Herein, bacterial microbiota of the female reproductive tract (environment) will be considered in the context of pregnancy outcomes where the host is the fetus and dam.

Female reproductive tract microbiome

Role of vaginal and uterine microbiome

Interactions between host and bacteria it supports has long been recognized; however, many questions remain regarding how this microbial environment varies within and among individuals in both healthy and diseased states.[3] Human vagina is colonized with commensal bacteria, predominantly *Lactobacilli*. Therefore, a vaginal microbiota has been proposed to have a role in female reproductive physiology, pathogen defense, and function.[4] Acidic environment created by these *Lactobacillus* spp. is thought to provide the first barrier of defense protecting upper reproductive tract from opportunistic pathogens in women.[3,4] A meta-analysis provided compelling data in women to support a uterine microbiome that may function to modulate the local immune system in preparation for embryo implantation and placenta formation.[5]

When comparing endometrial microbiota with vaginal and cervical microbiota in women, endometrial microbiota was in general lower in relative abundance, but with an overall higher microbial diversity and richness.[4,6] Recently, canine vaginal and uterine microbiome was published.[7] Metagenetic characterization of canine vagina and uterus was obtained from vaginal swabs and endometrial tissue samples, respectively, at elective ovariohysterectomy. Interestingly, top 5 genera identified in the vagina consisted of *Hydrotalea*, *Ralstonia*, *Mycoplasma*, *Fusobacterium*, and *Streptococcus* while top 5 genera identified in the endometrium included *Pseudomonas*, *Staphylococcus*, *Corynebacterium*, *Anaplasma*, and *Dermacoccus*. Vagina of bitch is higher in richness in contrast to canine endometrial tissue being higher in diversity. Vagina of estrual bitch had a distinct microbiome, whereas all other samples did not distinctly form clusters, regardless of stage of the estrous cycle. A study using multiple sampling techniques revealed that equine endometrium during estrus has a *Proteobacteria* driven microbiome, with no significant difference between vaginal and endometrial microbiomes.[8] Also, there were no significant differences between sampling techniques: endometrial double-guarded swab, low volume lavage, or endometrial biopsy.[8] In contrast, in another study, *Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria* in cycling equine endometrium was in contrast to vaginal microbiome.[9] However, equine endometrial microbiome does appear to be different in noncycling mares when compared to cycling mares during the physiologic breeding season (unpublished data). When making conclusions regarding female reproductive tract microbiome it is important to consider the stage of estrous cycle, which has unique sex steroid hormone influences, or lack thereof, on the immune milieu and potentially resident microbiome.

Further studies are necessary to determine a "core" vaginal and uterine microbiome that is advantageous for pregnancy success in all species, including dogs. With identification of canine reproductive tract microbiome, small shifts in microbiomes can be identified that may not be clinically apparent or previously detectable using conventional culture techniques, but could be clinically important.

Evidence for a canine placental microbiome

In both women and veterinary species, placenta has been regarded as a sterile organ during pregnancy. Placenta is responsible for maternal-fetal exchanges of nutrients and waste products, as well as tolerance of semi-allogeneic fetus that is composed of maternal and paternal antigens.[2] Successful placental development and fetal survival relies on immunotolerance by maternal uterine environment, which we are learning is more diverse and richer than previously thought. It was recently (2014) reported that, based on metagenetic techniques, human placenta harbors a unique microbiome.[3] Since then, nearly 100 new studies have been published on the human placental microbiome and metagenetic data are being generated in several veterinary species, including cows, dogs, and horses, that support a unique placental microbiome similar to that in women.

Taxonomic profile of human placental microbiome was discovered to be most similar to oral microbiome, followed closely by vaginal microbiome and further from fecal microbiome. Bacterial DNA was detected in only 50% of human placental tissue samples.[10] Based on these positive samples, healthy human placental microbiome has an abundance of *Firmicutes, Tenericutes, Proteobacteria,*

Bacteroidetes, and Fusobacteria.[11] Despite profound differences in placentation, this finding was consistent in horses.[1] Metagenetic analyses of the equine placenta, as done in humans, demonstrated 3 main phyla in the gravid horn (*Firmicutes, Proteobacteria, Bacteroidetes*) and those same 3 phyla plus Actinobacteria in the nongravid horn.[12] Most abundant bacterial phyla in gravid and nongravid chorioallantois share significant overlap, suggesting similar, but not identical, environments within different compartments of the chorioallantois. Many of the same bacterial populations that characterized gravid chorioallantois described by the Cornell University group were also identified in allantoic fluid of pregnant mares by investigators at Oklahoma State University.[9]

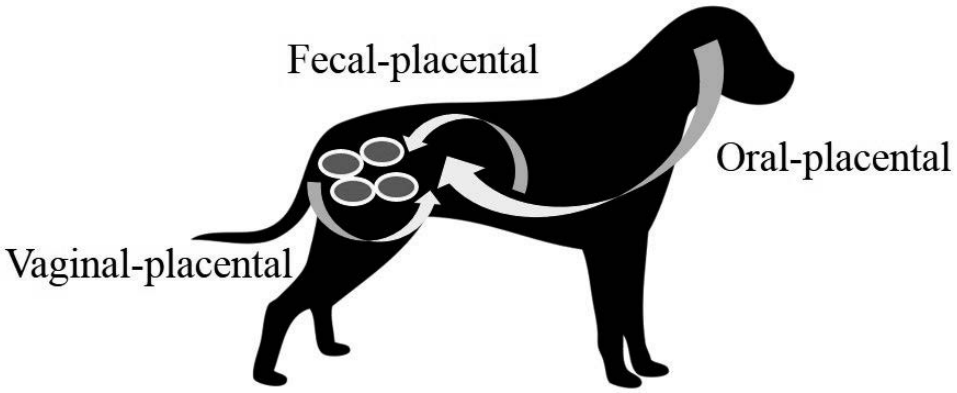

Figure. Potential extra-placental bacterial sources (oral, fecal, vaginal microbiota) for canine placental microbiome; canine fetoplacental units (pink).

It has been well established that there is an association between bacterial infection of reproductive tract and fertility.[13] Intense research efforts are focused on investigating bacterial endometritis in mare to improve fertility and pregnancy rates.[14] In dogs, relationship between endometritis and conception and/or pregnancy failure remains unclear.[15] Transcervical catheterization by vaginal endoscopy can be performed to evaluate both cytology and bacteriology. In a study where infertile bitches were examined using transcervical catheterization, 70% had heavy bacterial growth when samples were cultured *in vitro*. The most common bacteria include *Pasteurella multocida,* Group G *Streptococcus, Staphylococcus intermedius, Escherichia coli* and *Proteus mirabilis.*[15] These pathogens could all potentially contribute to a subclinical endometritis and even pyometra that may alter fertility as well as adverse pregnancy outcomes including abortion, stillbirth of preterm puppies, and neonatal loss. Reason why some bitches become pregnant and others do not is hypothesized to be due to the immune milieu of uterus, which may be influenced by a resident microbiome (Figure).

Importantly, associations between placental microbiome communities and adverse pregnancy outcomes in women, including preterm birth and preeclampsia, are rapidly being confirmed.[3,6] These new data can serve to prevent maternal and fetal morbidity and mortality via a novel mechanism, i.e. restoring a "healthy" microbiota prior to pregnancy. Preterm birth and neonatal death has been associated with intrauterine infections ascending from the lower genital tract.[7] In dogs, reproductive tract consists of a rich and diverse microbial environment with > 300 OTUs of organisms identified.[16] Similar to humans, presence of certain microbiota are either positively or negatively associated with pregnancy outcome. Presence of the any of the genera *Biberstinia, Staphylococcus, Pasteurella, Corynebacterium,* or *Methylobacterium* in vagina of bitch significantly increases the chance of a stillborn puppy in a litter.[17] Thus, this emerging and exciting new field supports a role for a symbiotic microbiome at maternal-fetal interface that contributes to pregnancy maintenance and success.

Novel metagenetic analyses of the canine placenta will be important to enhance our understanding of placental function during pregnancy in the bitch. Establishment of a "healthy"

reproductive tract microbiome could improve pregnancy outcomes in sub/infertile bitches. Furthermore, analysis of canine placental microbiome in relation to other extra-placental body sites in healthy bitches, similar to what has been investigated in women and mares, will provide foundation for a better understanding of the relationship between canine placenta and resident bacterial populations during uncomplicated healthy and complicated pregnancies.

Methods to characterize canine placental microbiome by metagenetics

To properly assess the canine placental microbiome, samples should be collected sterilely at the time of caesarian section. Collection of fetal membranes after a vaginal whelping will have contamination from the vagina. Canine endometrium and vagina have distinctly different microbiomes, collecting conceptuses that traverse vaginal vault will likely result in both microbiomes being represented. Genomic DNA can be extracted from various types of samples, such as swabs, fluid or tissue using commercial DNA isolation kits. After DNA extraction and purification, sample can be analyzed using multiple molecular genetic techniques. These involve isolation of 16S ribosomal DNA and sequencing of amplified bacterial DNA. Therefore, metagenetic techniques yield live and dead bacteria populations. Although dead and fragmented DNA is not replicating within a host, they still represent ligands for host cells to recognize and act upon. Therefore, these inactive bacterial fragments could potentially still contribute to a physiologic interaction with the host.[5] A second pitfall includes quantification of an organism present, despite available read counts.[18]

It is important to know that while traditional identification of bacteria utilizes cultivating methods (i.e. aerobic and anaerobic culture), metagenetic techniques utilize non-cultivating methods. Metagenetic analysis of placenta in horses should therefore be superior to cultivating methods that may fail to identify dormant bacteria that may reside at the maternal-fetal interface in pregnant mares. However, until these new methodologies are robustly tested and validated, the clinician should perform cultivating until non-cultivating methods are routinely available and used.

An outline of a suggested method to prospectively collect, process, and analyze canine placenta and extra-placental sites by metagenetic techniques is as follows:

Sample collection

To characterize the canine placental microbiome, vaginal swab samples as well as collection of transcervical catheters for endometrial metagenetic sequencing will be first collected during routine breeding management. In addition, swabs of the buccal mucosa of the oral cavity and a fresh fecal sample will be collected and stored at -80°C. At the time of pregnancy confirmation, repeat swabs of the vaginal vault and from the buccal mucosa of the oral cavity, and a fresh fecal sample will be collected and stored at -80°C. At the time Caesarean section delivery, placenta including marginal hematomas and amnion will be collected, dissected and stored at -80°C.

DNA isolation and Amplification of 16S rRNA

Frozen samples can be thawed and individually processed for genomic DNA extraction. This will be performed according to standard protocols. Samples homogenized, centrifuged and resuspended in nuclease-free water. An equal volume of lysozyme added and incubated for 12 hours at 56°C to maximize bacterial DNA extraction. Isolation of DNA performed using a commercial DNA isolation kit PowerSoil® (Mo-Bio Laboratories Inc) according to the manufacturer's instructions. DNA concentration determined using the Qubit® 2.0 Flurometer (Life technologies, Grand Island, NY) and DNA integrity assessed by electrophoresis. The V3 and V4 domain of bacterial 16S rRNA[8] amplified by PCR. Primer sequence for 16S Amplicon PCR is the 341F and 785R primers as previously described[9] and optimized for the Illumina MiSeq platform. Earth microbiome project (http://www.earthmicrobiome.org)[10] can be used to select 150 different 12 bp error-correcting Golay barcodes for 16S rRNA, as described.[9] The PCR reaction performed in triplicate containing: microbial genomic DNA (25 ng), 1x GoTaq Green Master Mix (Promega, Madison, WI), 1 mM magnesium chloride, and 10 μM of each primer. The PCR conditions for 16S rRNA consist of an initial denaturation step of 3 minutes at 94°C; followed by 35 cycles of 94°C for

45 seconds, 50°C for 1 minute, and 72°C for 90 seconds; and a final elongation step of 72°C for 10 minutes. No template controls and 2 positive controls (mock microbial communities) should be added to each PCR plate. Replicate amplicons pooled and purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA) and visualized by electrophoresis. Amplicons are quantified using Qubit® 2.0 Flurometer then the amplicon aliquots are standardized to the same concentration.

MiSeq Sequencing

Final equimolar libraries will be sequenced using the MiSeq reagent kit V3 (600 cycles) on the MiSeq platform (Illumina Inc., San Diego, CA). Sequence reads analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) program and assigned to OTUs. Discriminant analysis is used to evaluate the correlation between bacterial taxa and the prevalence of each bacteria identified in the placental samples against the potential source of bacteria from extra-placental body sites. Different prevalence of bacteria in each sample will be used as a covariate in a stepwise discriminant analysis model. Variables removed in a stepwise manner until the only variable with a $p < 0.005$ is retained in the final model.

Significance

Preterm birth of stillborn puppies is not an uncommon outcome of pregnancy in dogs. Diagnosing the cause of canine abortion is best done through necropsy of fetuses and fetal membranes. Placental infection during canine pregnancy can be extremely difficult to diagnose, as outward clinical signs are often not present or subtle, consisting of restlessness, vulvar discharge, and lethargy. Understanding relationship between canine placenta and resident bacterial populations of uterus and vagina could provide opportunity to establish symbiosis prior to pregnancy in order to aid in pregnancy success. Furthermore, diagnosing dysbiosis in uterus before pregnancy or even extra-placental sites (oral, gastrointestinal and/or vaginal) could provide evidence for predicting associated adverse outcomes, such as abortion, in breeding bitches. Identification of bacterial targets in extra-placental body sites and appropriate pre/probiotic therapies to improve adverse pregnancy outcomes would greatly enhance breeding management and perinatal care in our canine patients. Authors advise caution and judicious usage of interventions to alter the microbiome of canine reproductive tract to improve fertility and pregnancy outcomes until an exhaustive microbiota characterization has been performed.

Conflict of interest

No conflict of interest to declare.

References

1. Concannon PW: Canine Pregnancy: Predicting Parturition and Timing Events of Gestation. IVIS 2000.
2. Furukawa S, Kuroda Y, Sugiyama A. A comparison of the histological structure of the placenta in experimental animals. J Toxicol Pathol 2014;27:11-18.
3. Green KA, Zarek SM, Catherino WH: Gynecologic health and disease in relation to the microbiome of the female reproductive tract. Fertil Steril 2015;104:1351-1357.
4. Wee BA, Thomas M, Sweeney EL, et al: A retrospective pilot study to determine whether the reproductive tract microbiota differs between women with a history of infertility and fertile women. Aust N Z J Obstet Gynaecol 2018;58:341-348.
5. Benner M, Ferwerda G, Joosten I, et al: How uterine microbiota might be responsible for a receptive, fertile endometrium. Hum Reprod Update 2018;24:393-415.
6. Verstraelen H, Vilchez-Vargas R, Desimpel F, et al: Characterisation of the human uterine microbiome in non-pregnant women through deep sequencing of the V1-2 region of the 16S rRNA gene. PeerJ 2016;4:e1602.
7. Lyman CC, Holyoak GR, Meinkoth K, et al: Canine endometrial and vaginal microbiomes reveal distinct and complex ecosystems. PLoS One 2019;14:e0210157.
8. Heil BA, Thompson SK, Kearns TA, et al: Metagenetic characterization of the resident equine uterine microbiome using multiple techniques. J Equine Vet Sci 2018;66:111.
9. Holyoak GR, Lyman CC, Wieneke X, et al: The equine endometrial microbiome. Clinical Theriogenology 2018;10:273.

10. Doyle RM, Harris K, Kamiza S, et al: Bacterial communities found in placental tissues are associated with severe chorioamnionitis and adverse birth outcomes. PLoS One 2017;12:e0180167.
11. Aagaard K, Ma J, Antony KM, et al: The placenta harbors a unique microbiome. Sci Transl Med 2014;6:237ra65.
12. Xia YX, Cornelius AJ, Donnelly CG, et al: Metagenomic analysis of the equine placental microbiome. Clinical Theriogenology 2017;9:452.
13. Canisso IF, Stewart J, Coutinho da Silva MA: Endometritis: Managing Persistent Post-Breeding Endometritis. Vet Clin North Am Equine Pract 2016;32:465-480.
14. Ferris RA: Endometritis: Diagnostic Tools for Infectious Endometritis. Vet Clin North Am Equine Pract 2016; 32:481-498.
15. Fontaine E, Levy X, Grellet A, et al: Diagnosis of endometritis in the bitch: a new approach. Reprod Domest Anim 2009;44 Suppl 2:196-199.
16. Lyman CC, Meinkoth K, DeSilva U, et al: 16S rDNA-based profiling of canine reproductive tract microbiota reveals a complex microbial ecosystem. Clinical Theriogenology 2016;8:305.
17. Cornelius AJ, Bichalho RC, Cheong SH: The canine vaginal microbiome and associations with puppy survival. Clinical Theriogenology 2017;9:424.
18. Bracewell-Milnes T, Saso S, Nikolaou D, et al: Investigating the effect of an abnormal cervico-vaginal and endometrial microbiome on assisted reproductive technologies: A systematic review. Am J Reprod Immunol 2018;80:e13037.