Equine placental microbiome

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Introduction

The mammalian placenta has long been regarded as a sterile organ that nourishes and protects the growing fetus during gestation. There is recent evidence in mares that the placenta harbors a unique microbiome.[1] While this work is in its infancy in the horse, there is a growing amount of data in human literature to support the role of the placental microbiome in pregnancy success and adverse outcomes. The following paper will review and highlight this up and coming area of interest in equine reproduction.

Background

The placenta is a vital, but transient organ of pregnancy that ensures fetal growth and survival until the time of delivery. It is composed of fetal membranes in intimate association with the mother's uterus. Placental development and fetal survival relies on tolerance by the maternal environment. The placenta is responsible for maternal-fetal exchanges of nutrients and waste as well as tolerance of the semi-allogeneic fetus that is composed of maternal and paternal antigens.[2] The placenta also provides pregnancy maintenance by producing a number of pro-gestational hormones. Thus, it's immunologic and endocrinologic functions have been well characterized and studied. In both women and mares, the placenta has been thought of as a sterile organ during pregnancy. However, it was recently elucidated in women that the placenta harbors a unique microbiome and this was discovered using molecular biology, specifically metagenetic, techniques.[3] As a result, a potential new role of the placenta during pregnancy has been proposed. The taxonomic profile of the human placental microbiome was discovered to be most similar to the oral microbiome followed closely by the vaginal microbiome and further from the fecal microbiome.[3] This finding was found to be consistent in horses and the results are described below.[1]

There is strong rationale to support challenging the dogma of a sterile placenta in horses. Distinct uterine microbiomes have been reported between diestrual mares (Day 7 after ovulation) carrying an embryo and those that are open using metagenetics. Proteobacteria and Bacteroidetes were associated with culture positive samples at ovulation. Sphingobium (Proteobacteria) and Sphingobacteriales (Bacteroidetes) are associated with mares carrying embryos at Day 7 after ovulation and Rhodocyclaceae and Enterobacteriaceae (Proteobacteria) are associated with mares not carrying embryos.[4] Also, amniotic fluid taken at delivery from healthy equine pregnancies has yielded bacterial growth.[5] Additionally, foal meconium on postnatal day 1, prior to suckling, is dominated by the Firmicutes phyla consisting primarily of the genera *Enterococcus, Bacillus* and *Lactococcus*. There were no differences between mare milk and foal fecal bacterial diversity in meconium or feces from postnatal day 2 and 7.

Recent evidence suggests there are associations between placental microbiome communities and adverse pregnancy outcomes in women, including preterm birth and preeclampsia.[3,6] Indeed, preterm birth in people has been associated with intrauterine infections ascending from the lower genital tract as well as intra-amniotic and extra-uterine infections.[7] While the vaginal lactic acid bacteria have been evaluated in the mare by traditional culture methods, metagenomic sequencing of the vaginal microbiome in the context of healthy pregnancies and adverse pregnancy outcomes has not yet been reported in the mare.

Novel metagenetic analyses of the equine placenta, similar to that done in humans, in relation to other extra-placental body sites in healthy mares, will provide the foundation for a better understanding of the relationship between the equine placenta and resident bacterial populations during uncomplicated healthy pregnancies.

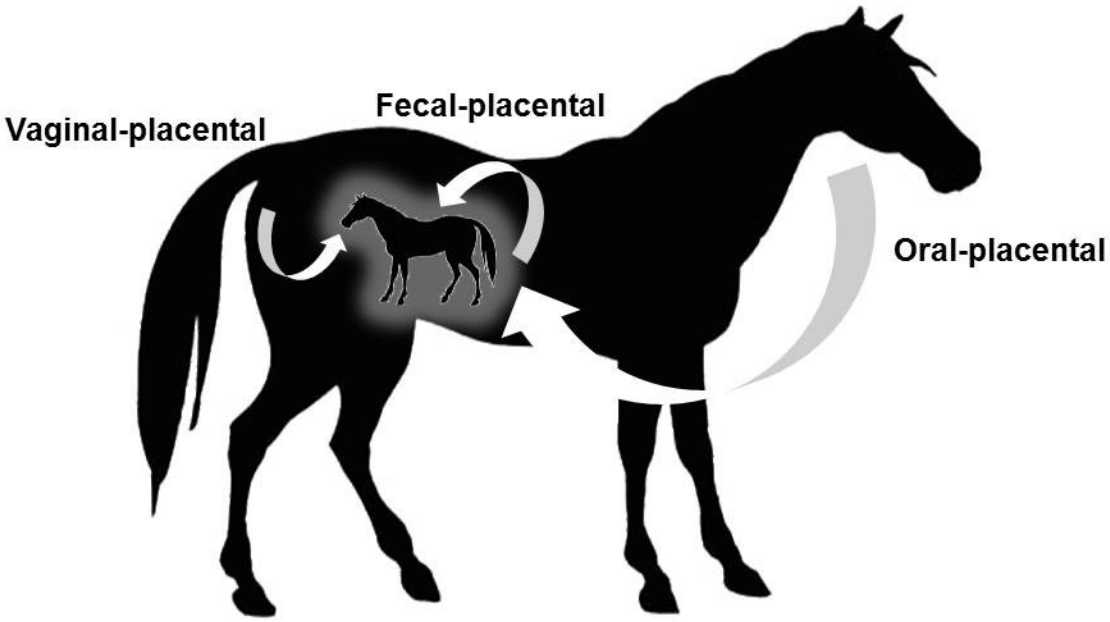

Figure. The equine fetoplacental unit in relation to profiles of extra-placental bacterial populations associated with equine placentitis (oral, fecal, vaginal).

Methods to characterize the equine placental microbiome

The microbiome consists of the microorganisms and their genomes within a specific region of the body. 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. These involve isolation of 16S ribosomal DNA and sequencing of the amplified bacterial DNA. Therefore, metagenetic techniques yield live and dead bacteria populations. Metagenetic analysis of the 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.

To characterize the first equine placental microbiome, university owned mares were used for sampling prior to and at the time of foaling (n=15).

Collection of samples

Within thirty days of foaling, swabs from the buccal mucosa of the oral cavity, the cranial vaginal using sterile instruments and speculum, and a fresh fecal sample were collected and stored at -80°C. At the time delivery, placenta and specific regions of fetal membranes (gravid and non-gravid chorioallantois) were collected, dissected and stored at -80°C.

DNA isolation and amplification of 16S rRNA

Frozen samples were thawed and individually processed for DNA extraction. Samples were homogenized, centrifuged and resuspended in 400 µl of nuclease-free water. Four hundred µg of lysozyme was added and incubated for 12 hrs at 56°C to maximize bacterial DNA extraction. Isolation of DNA was performed using a commercial DNA isolation kit PowerSoil® (Mo-Bio Laboratories Inc) according to the manufacturer's instructions. DNA concentration was 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] was amplified by PCR. Primer sequence for 16S Amplicon PCR was the 341F and 785R primers as previously described[9] and optimized for the Illumina MiSeq platform. The earth microbiome project (http://www.earthmicrobiome.org)[10] was used to select 150 different 12 bp

error-correcting Golay barcodes for 16S rRNA as previously described.[9] The PCR reaction was performed in triplicate containing: microbial genomic DNA (25ng), 1x GoTaq Green Master Mix (Promega, Madison, WI), 1 mM magnesium chloride, and 10 μM of each primer. The PCR conditions for 16S rRNA consisted of an initial denaturation step of: 3 min at 94°C; followed by 35 cycles of 94°C for 45 secs, 50°C for 1 min, and 72°C for 90 secs; and the final elongation step of 72°C for 10 min. No template controls and two positive controls (mock microbial communities) were added to each PCR plate. Replicate amplicons were pooled and purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA) and visualized by electrophoresis. Amplicons were quantified using Qubit® 2.0 Flurometer then the amplicon aliquots were standardized to the same concentration.

MiSeq sequencing

Final equimolar libraries were sequenced using the MiSeq reagent kit V3 (600 cycles) using the MiSeq platform (Illumina Inc., San Diego, CA). Sequence reads were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) program and assigned to Operational Taxonomic Units (OTUs). These OTUs identify the genera of bacteria present in the sample. Discriminant analysis (JMP Pro 12) was used to evaluate the correlation between bacterial taxa and the prevalence of each type of bacteria in the placental samples with the potential source of bacteria from extra-placental body sites. Different prevalence of bacteria in each sample was used as a covariate in a stepwise discriminant analysis model. Variables were removed in a stepwise manner until the only variable with a $p < 0.005$ were retained in the final model.

Results

Relative abundance within the chorioallantois demonstrated three main phyla represented in the gravid horn (Firmicutes, Proteobacteria, Bacteroidetes) and those same three phyla plus Actinobacteria in the non-gravid horn. The most abundant phyla within the oral, fecal, and vaginal samples (Firmicutes and Proteobacteria) were also detected in the chorioallantois. The gravid horn and feces of the mare have different populations of bacteria. *Bacillus, Mycoplasma,* and *Gemella* are all higher in relative abundance in the fecal samples of the mare ($p<0.0001$). Conversely, *Clostridium* and *Moraxella* are higher in the gravid horn samples ($p<0.0001$). The most abundant bacterial phyla in gravid and nongravid chorioallantois share significant overlap, suggesting similar, but not identical, environments within different compartments of the chorioallantois. Studies are still in progress to evaluate the cervical star region of the chorioallantois, which is a key area of the equine placenta where ascension of bacteria from the lower reproductive tract would occur.

Discussion

Placentitis is the most common cause of equine preterm birth in the United States and accounts for one-third of all late-term abortions/fetal loss of horses in this country.[11] The most common cause of placentitis is bacteria ascending from the lower reproductive tract initiating an infectious inflammatory response in the placenta.[12] Oral and fecal pathogens (ie *Streptococcus equi* subspecies *zooepidemicus*, *Esherichia coli*) are often cultured in cases of equine placentitis, but it is not clear why some mares are susceptible to these organisms and others are not. Furthermore, diffuse bacterial placentitis cases, such as Nocardioform, do not follow the same pathogenesis of ascending placentitis cases. Therefore, the mechanism needs further elucidation. Future direction would include evaluation of the vaginal microbiome in mares. It has been shown by cultivating methods that the vagina of the mare is rich in *Lactobacillus* and *Enterococcus* species.[13] This is of particular interest as *Lactobacillus cispatus* has been used with a success rate of 69-90% when used to colonize vaginas of sexually active healthy women seeking assisted reproductive techniques. Colonizing the transfer-catheter tip with *Lactobacillus crispatus* at the time of embryo transfer may increase the rates of implantation and live birth rate while decreasing the rate of infection.[14]

Phyla of relatively high abundance in oral and vaginal samples correspond to those found in the chorioallantois, indicating possible associations between placental and extra-placental microbiota, yet

there are significant differences between the gravid horn and the fecal samples. To the authors' knowledge, this is the first report to characterize the equine placental microbiome by metagenetics. Further studies include pyrosequencing of equine placentae from mares with adverse pregnancy outcomes, i.e. placentitis and fetal growth restriction, to determine if the equine placenta has a unique microbiome in health and disease.

Several maternal risk factors are known or hypothesized to impact the human placental microbiome, including obesity/excessive gestational weight gain, diabetes, and even periodontal disease.[15] As this area of research grows, it will be important to analyze equine placental microbiomes in the context of overall mare health status before and during pregnancy. This knowledge will also help guide the way we diagnose and treat dysbiosis of the mare's reproductive tract in an effort to prevent adverse pregnancy outcomes in the horse. This author advises caution and judicious usage of interventions to alter the microbiome of the mare's reproductive tract until an exhaustive microbiota characterization has been performed.

Significance

Diagnosing placental infection during equine pregnancy is extremely difficult since outward clinical signs are often not present. Understanding the relationship between the equine placenta and resident bacterial populations during healthy and diseased pregnancies could provide the opportunity to use extra-placental sources (oral, fecal and/or vaginal) as "proxies" for predicting placentitis and associated adverse outcomes. Further investigations are required to evaluate clinical cases of equine placentitis as well as the mare's uterine microbiome before pregnancy. Identification of bacterial targets in extra-placental body sites as causative in adverse pregnancy outcomes would revolutionize the way we manage pregnancy in the mare.

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