

## The equine endometrial microbiome

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### Context

Our purpose is to examine an alternative idea to describing the reproductive tract microbiome in the mare. Defining the reproductive tract microflora in any species including the mare lies in the need to be able to determine the physiologic state of the microbiome in healthy patients in order to develop a deeper understanding of the impact dysbiosis can have on overall health. With the background information we have, and the advancement of sequencing techniques at our disposal it may be time to challenge the veterinary dogma of a “sterile uterus” and adjust our clinical practices accordingly.

**Keywords:** Mare, paradigm shift, endometrial microbiota, 16S rDNA

### Introduction

It has been standard dogma that although the vagina is colonized with commensal bacteria, the uterus is maintained as a sterile environment<sup>1</sup> and a failure to eliminate bacteria, sperm and inflammatory products from the uterus after breeding will result in endometritis and reduced fertility.<sup>2</sup> While relatively more is known about the microbiome of the human reproductive tract, much less is known about the microbial communities residing in the reproductive tract of animals.

As with the mare, in cattle it has long been held that bacterial contamination during the periparturient period lead to endometritis or metritis in the cow.<sup>3</sup> In all of our farm animal species these dogmata were based on the results from media based culture systems.

Although culture-based studies have laid out the foundation of our understanding of the uterine microbiota, almost three decades ago there was an indication that culture based methodologies in identifying microbial populations may underestimate diversity and overestimate the role of culturable bacteria.<sup>4</sup> Focusing on culturable bacteria, often the rarer members of microbial communities, enhances the risk of missing those microbes that are more abundant.<sup>5,6</sup> Recently we completed a study in the canine reproductive tract documenting with 16s rDNA based metagenomics the presence and diversity of both vaginal and uterine microbiomes at the various stages of the estrous cycle, concluding that both the endometrium and the vagina have rich microbial ecosystems.<sup>7</sup> Additionally, a study utilizing culture-independent 16S ribosomal RNA (rRNA) sequencing of the bovine and ovine vaginal microbiota from ectocervicovaginal lavages revealed that cow and ewe vaginal microbiota are unique from previously described vaginal microbial ecosystems.<sup>8</sup> It is clear from these studies that culture-based diagnostic systems miss the great diversity present in both diseased and healthy reproductive tracts and express a need for metagenomic analytics.

We began our metagenomics investigations in the mare to document the underestimation of microbial diversity with common culture methods relative to the presence of microbial DNA using the approach six years ago.<sup>9</sup> This study grew from a study utilizing molecular identification performed by sequencing of PCR products of 16S rDNA using 63F and 1389R primers which suggested separate populations of microbiota between the equine uterus and vagina.<sup>10</sup> Differences were shown between vaginal and uterine microbial populations. That while there was a demonstrated transfer of a vaginal subpopulation into the uterus post-breeding there remained distinct differences between the vaginal and endometrial microbiomes.<sup>10</sup> However, neither of these studies clearly defined the endometrial microbiota.

The purpose of describing the reproductive tract microflora in any species including the mare lies in the clinical need to be able to determine the physiologic state of the microbiome in healthy patients in order to develop a deeper understanding of the impact dysbiosis can have on overall health. Understanding the ramifications that broad-spectrum antibiotic use can have on the uterine normal flora should dictate to veterinarians and breeders that treating a breeding mare with antibiotics, in the absence of an understanding of the endometrial microbiome may be counterproductive. With the background

information we have and the advancement of sequencing techniques at our disposal it is time to more deeply investigate the dogma of a “sterile uterus” and adjust our clinical practices accordingly if needed.

The purpose of this report is to discuss what we have learned in our efforts to characterize the equine microbiome in the non-pregnant mare.

## Materials and methods

### Animals

Twenty nine mares between the age of 8 and 18 years of age, with no history of reproductive problems were used in the study. The mares were given a complete pre-study breeding soundness examination to include only those without clinical signs of endometritis.

### Small volume lavage (SVL)

All mares had uterine luminal endometrial microbiota samples collected via 150 ml of saline infused via a double guarded system, wherein the first guard is inserted into the cervix and an internal tube containing a catheter is advanced into the uterus, saline is infused, massaged within the lumen and aspirated back and the tubing then retracted before removing back through the vaginal vault. This sampling technique has been shown to have the highest return rate for microbial collection.<sup>11</sup>

### Microbiota analysis

Small volume lavage samples were spun down to collect bacterial cells and total DNA from the sediment extracted using a commercial DNA isolation kit (QIAmp DNA mini kit, Qiagen, Germantown, MD) and following manufacturer’s instructions. A ~250 bp fragment from ribosomal v4 region was PCR-amplified from resulting DNA. Amplicon sequencing was performed by Molecular Research LLC (Mr DNA), Shallowater, TX using their established protocols.<sup>12</sup> Briefly, a library was built using TruSeq® DNA PCR-free sample preparation kit (Illumina Inc., San Diego, CA) following instructions and index codes were added. The library was quality tested on a Qubit 3.0 fluorometer (ThermoScientific, Waltham, MA) and an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). The library was sequenced on an Illumina HiSeq2500 platform (Illumina) and 250bp paired-end raw reads were generated.

### Sequence analysis

Mothur 1.37 was used for data mining following the MiSeq standard operating procedure (SOP).<sup>13</sup> Briefly, paired-end reads were assembled and assigned to each sample based on their unique barcode and then truncated by removing barcodes and primer sequences. Reads that were over 273 bp in length, those that contained homopolymeric tracts of longer than 8 bp in length, those that had more than one mismatch against primer sequences, contained undetermined bases, ambiguities, or did not align to V4 hypervariable region were removed from the analysis. Alignment of V4 region against SILVA rRNA ref nr123<sup>14</sup> was performed using mothur (Needleman-Wunsch algorithm). Uchime was used to remove chimeric sequences.<sup>15</sup> The rest of the analyses were performed following mothur MiSeq standard operating procedures. Sequences were assigned to operational taxonomic units (OTUs) based on 97% similarity.

### Statistical analyses

Neighbor-joining trees were generated using MEGAN Community Edition.<sup>16</sup> The Shannon diversity index was calculated using mothur and principal coordinate analyses (PCoA) was calculated based on the distance matrix.

## Results

1.1 million sequence reads of V4 region of 16S rRNA gene were generated in this study with an average of ~40,000 reads per mare. Upon analysis, we identified 160 unique genera of bacteria belonging

to 18 different phyla. There were significant differences among animals with some clustering based on the source of animals. We defined a core microbiome for equine uterus at phylum level as phyla that were present at least at 1% level in all samples where they were identified and present in at least 90% of samples. The core microbiome at phylum level across all samples were; Proteobacteria (100%), Firmicutes (100%), Bacteroidetes (96.2%) and Actinobacteria (100%). We defined the core microbiome at genus level as present in at least 60% of the samples and are present at least at 0.5% in samples where the organism is detected. The core microbiome at genus level across all samples were; *Pseudomonas* (100%), *Porphyromonas* (73.1%) and *Streptococcus* (61.4%). There were animal source specific core microbiomes.

## Discussion

The mammalian vagina is known to harbor a rich microbial ecosystem, while the human vaginal microbiome is well-described,<sup>17-23</sup> only a few non-human vaginal microbiomes have been described to date.<sup>8,24-26</sup> The endometrium on the other hand, was considered a sterile environment<sup>1,27</sup> until a few years ago when that paradigm was challenged by the observation that the human placenta harbors a small but diverse microbiome.<sup>27,28</sup> This new view is, however, not without debate.<sup>29</sup> In addition to the human work, a few studies have been performed exploring the microbiome of the bovine and ovine endometrium and placenta.<sup>30-33</sup> While the microbiota of the cow and ewe were similar, the bovine vaginal microbiome showed greater diversity compared to the ovine and both were considerably different from those reported in the human and non-human primates.

Last year Schnobrich, et al. described “Next Generation Sequencing” (MicroGenDX, formally PathoGenius Laboratory, Lubbock, TX) of samples from 10 clinically normal mares and compared those results to traditional methods of diagnosing infectious endometritis.<sup>34</sup> The main problem with the analyses is that the DNA sequences were compared against a bank of known human and equine pathogens, therefore they did not consider all possible normal non-culturable bacteria that have heretofore been undocumented in the normal microbiota of the mare.

Also last year a second abstract was presented very similar to one we published in 2011<sup>9</sup> wherein uterine fluid samples obtained from mares were subjected to metagenomic DNA sequencing of the 16S rRNA gene. These mares were followed around the time of ovulation/artificial insemination (n=10) and during early pregnancy (n=10). The metagenomic sequencing identified over 200 bacterial species in both culture negative and culture positive samples demonstrating as we have that the uterus is not a sterile site at any point during and after estrus. Proteobacteria and Bacteroidetes phyla were statistically associated with culture positive samples according to the Bonferroni correction. Their pilot study strongly correlates with ours in evidencing of the presence of a complex bacterial microbiome of organisms that fails to grow using routine uterine culture methods.<sup>35</sup>

Finally, from the same proceedings a third abstract was published describing the Metagenomic analysis of the equine placental microbiome. Fecal, oral, and vaginal samples were taken from pregnant mares within 30 days of foaling, as well as the gravid and non-gravid regions of the chorioallantois at the time of foaling (n=4). Genomic DNA was isolated from all samples, and the bacterial 16s ribosomal RNA gene was amplified by PCR. Similarly they reported a relative abundance of bacterial species within the chorioallantois. The three phyla represented in the gravid horn were Firmicutes, Proteobacteria, Bacteroidetes, with the 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 authors noted that 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. They also reported that phyla of relatively high abundance in oral and vaginal samples corresponded to those found in the chorioallantois, indicating possible associations between placental and extra-placental microbiota. However they found significant differences between the gravid horn and the fecal samples.<sup>36</sup>

Clearly, this is a fast moving and clinically relevant area of investigation within the field of equine reproductive medicine and health. Further controlled studies are underway in determining the

viability of this diagnostic tool in identifying clinical and subclinical cases of infectious endometritis and the role of specific components of the microbiome in the promotion of fertility and maintenance of pregnancy. In this study we have provided the largest and most comprehensive analysis of equine uterine microbiomes to date and have established the core microbiome of the healthy uterine endometrium. We have also demonstrated that the uterine microbiome of mares raised at deferent geographical locations differ from each other.

## Conclusion

We conclude from our results as well as past studies that culture-based systems miss the great diversity present in both diseased and healthy reproductive tracts. Given this diversity, there is much to be studied relative to the intra-microbiota interactions and species-intrinsic factors that may be more relevant to the maintaining a state of balance and health and that an imbalance of these factors maybe more important in the development of uterine disease than the abundance of any given bacterial species.

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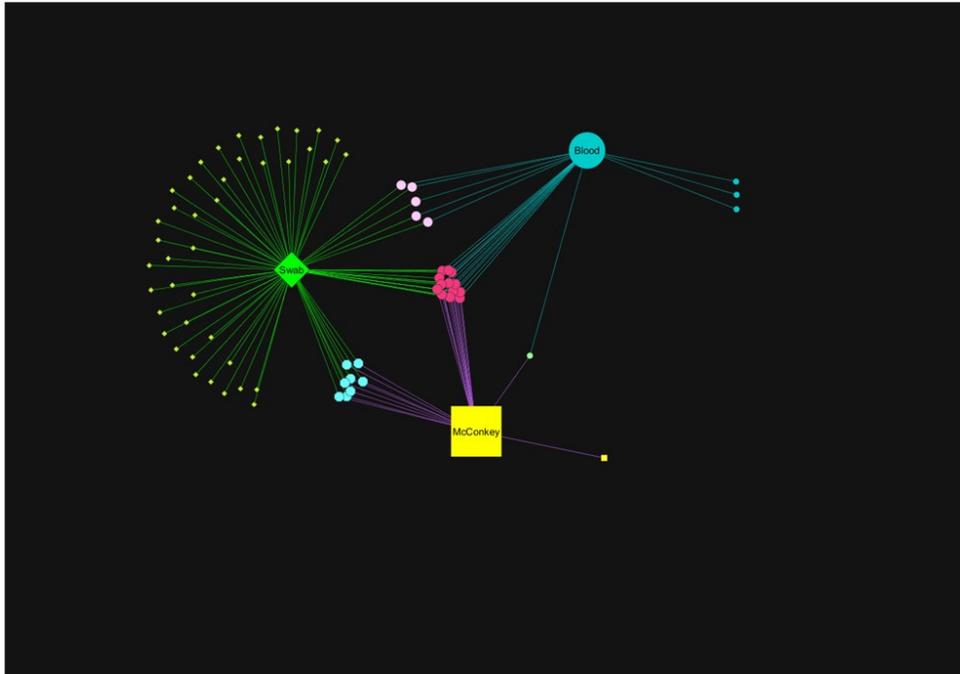


Figure. Microbes isolated from non-culturable methods significantly differ from culture-based methods. A uterine swab obtained from a single mare were either analyzed using direct 16S rDNA sequencing (Swab), or after culturing on McConkey (McConkey) or blood agar (Blood) plates. Each line denotes a novel OTU and those that are connected with two or more methods were co-identified by the linked methods.