

An Advanced Understanding of Uterine Microbial Ecology Associated with Metritis in Dairy Cows

Soo Jin Jeon^{1*}, Klibs N. Galvão^{2,3}

¹Department of Biomedical Veterinary Sciences, College of Veterinary Medicine, Long Island University, Brookville, NY 11548, USA, ²Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL 32610, USA, ³D. H. Barron Reproductive and Perinatal Biology Research Program, University of Florida, Gainesville, FL 32610, USA

Metritis, the inflammation of the uterus caused by polymicrobial infections, is a prevalent and costly disease to the dairy industry as it decreases milk yield, survival, and the welfare of dairy cows. Although affected cows are treated with broad-spectrum antibiotics such as ceftiofur, endometrial and ovarian function are not fully recovered, which results in subfertility and infertility. According to culture-dependent studies, uterine pathogens include *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, and *Prevotella melaninogenica*. Recent studies using high-throughput sequencing observed very low relative abundance of *Escherichia coli*, *Trueperella pyogenes*, and *Prevotella melaninogenica* in cows with metritis. Herein, we propose that metritis is associated with a dysbiosis of the uterine microbiota, which is characterized by high abundance of *Bacteroides*, *Porphyromonas*, and *Fusobacterium*.

Keywords: cattle, metagenomics, microbiota, uterine diseases

Introduction

Metritis is an acute inflammatory disease in the uterus of dairy cows, found in ~20% of lactating dairy cows, with the incidence ranging from 8% to more than 40% in some farms [1-3]. Metritis gives rise to a reduction in reproductive performance and milk production and an increase in culling and antibiotic use [4-6], which costs about \$358 per case of metritis [7]. Given the U.S. dairy cow population of 9.3 million [8] and the typical incidence in U.S. herds of 20%, the total cost of metritis to the US dairy industry is estimated at approximately \$665 million.

Uterine infection is a common phenomenon after parturition and thus uterine discharge is normal physiology for postpartum cows to eliminate bacteria residing in the uterus. However, when cows fail to naturally eradicate bacteria, there is a greater pathogen burden that causes them to suffer from inflammation with enlarged uterus and fetid red-brownish uterine discharge [5, 9]. A fever >39.5°C can accompany these symptoms, but not all of metritic cows show a fever [10, 11].

Escherichia coli, *Trueperella pyogenes*, *Fusobacterium necrophorum*, and *Prevotella melaninogenica* are thought to be primary pathogens causing uterine disease, because these bacteria were frequently isolated from the uterine lumen of diseased cows, and they were shown to affect endometrium and ovary function [12-17]. In particular, *E. coli* emerged in the uterus within a few days of calving, followed by the colonization of *T. pyogenes* and *F. necrophorum*, suggesting that the uterine pathogens work synergistically. However, it should be noted that these bacteria were mostly isolated in cows with endometritis, which is different from metritis in terms of time and location of infection. Previous studies based on a culture method also have a limitation of detecting fastidious anaerobic microorganisms that require specific nutrients for growth on media. Indeed, it has been suggested that about less than 1% of environmental microorganisms are cultivable in the laboratory [18].

To overcome limitations in culture methods, recent studies have used metagenomic sequencing which is a powerful tool to identify bacterial community. For example, clone library sequencing and pyrosequencing of the 16S rRNA gene revealed uterine bacterial community of dairy

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*Corresponding author: Tel: +1-516-299-3691, E-mail: soojin.jeon@liu.edu

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cows that was different between cows with and without metritis [19-22]. The Illumina platform allowed deeper sequencing than previously possible, with the potential to detect very rare phylotypes, which makes it possible to define uterine microbiota associated with metritis [10, 23-26]. Herein, the aim of the current paper is to review some recent studies that used metagenomic approaches to explore uterine bacterial community involved in metritis and discuss uterine bacteria that are known and newly identified in metagenomic sequencing.

Uterine Microbiota Associated with Metritis

As technology for pathogen detection has developed from culture method to next-generation sequencing, our knowledge about bacterial community structure and its influence on human health and disease is developing rapidly [27, 28]. Today, the advanced technology is helping bring to light uterine microbiota associated with metritis in dairy cows. An early study using denaturing gradient gel electrophoresis and 16S rRNA gene clone library sequencing determined that Fusobacteria was found to be more dominant in the metritic cows, and Gammaproteobacteria more dominant in healthy cows [19]. Another study examined the abundance and diversity of uterine microbiota in healthy and metritic cows using the clone library sequencing of 16S rRNA gene and quantitative polymerase chain reaction (PCR) analysis, and found that of all bacteria, Bacteroidetes, *Peptostreptococcus*, and *Fusobacterium* were

more abundant in metritic cows than in healthy cows on 10 days postpartum (DPP) [21]. Furthermore, metagenomic pyrosequencing allowed the comparison of relative abundance of each bacteria in samples from metritic and non-metritic cows, where *Bacteroides* spp. and *Ureaplasma* spp. were found to be associated with metritis [20]. In this study *Odoribacter* spp., *Peptoniphilus* spp. and *Helcococcus* spp. were reported to be associated with uterine health and reproductive performance. However, it should be noted that they collected samples on 35 ± 3 DPP after the metritis was resolved. Considering dynamic changes of uterine microbiota [22, 23, 25], it is difficult to conclude that these bacteria identified on 35 ± 3 DPP contributed to metritis. In short, early metagenomic studies using clone library sequencing and pyrosequencing enabled the discovery of diverse and abundant uterine microbiota of dairy cows, pointing out the difference in bacterial community structure between healthy and metritic cows. However, these studies were unable to define specific uterine microbiota associated with metritis due to the lack of statistical power and technical limit of detection.

To determine specific uterine microbiota associated with metritis, Jeon *et al.* [23] investigated uterine microbiota progression from calving (0 DPP) until establishment of metritis (6 ± 2 DPP) using Illumina sequencing of 16S rRNA genes. They observed that uterine microbiota structure was identical between cows that developed metritis and healthy cows until 2 DPP, when the bacterial structure deviated in favor of greater relative abundance of *Bacteroides*,

Table 1. Metagenomic analysis of uterine microbiota to identify pathogenic bacteria associated with metritis in postpartum dairy cows

Study	Bacteria (taxonomic categories)	Type of sample (DPP)	Analytical methods
Santos <i>et al.</i> [19]	Fusobacteria (phylum)	Uterine fluid	DGGE and 16S clone library sequencing
Machado <i>et al.</i> [20]	<i>Ureaplasma</i> (genus), <i>Bacteroides</i> (genus)	Uterine fluid (35 ± 3)	Pyrosequencing (V1-2)
Santos and Bicalho [22]		Uterine fluid (1-3, 8-10, 34-36)	DGGE and pyrosequencing (V1-2)
Peng <i>et al.</i> [21]	Bacteroidetes (phylum) <i>Peptostreptococcus</i> (genus) <i>Fusobacterium</i> (genus)	Uterine fluid (10)	qPCR and 16S clone library sequencing
Jeon <i>et al.</i> [10, 23, 24]	<i>Bacteroides</i> (genus) <i>Porphyromonas</i> (genus) <i>Fusobacterium</i> (genus)	Swab (6 ± 2)	Illumina MiSeq (V4)
Knudsen <i>et al.</i> [25]	Porphyromonadaceae (family) Fusobacteriaceae (family)	Uterine flush and endometrial biopsy (4-12)	Illumina MiSeq (V1-2)
Bicalho <i>et al.</i> [26]	Bacteroidetes (phylum) Fusobacteria (phylum)	Swab (3-12)	Illumina MiSeq (shotgun)
Sicsic <i>et al.</i> [29]	<i>Bacteroides</i> (genus) <i>Porphyromonas</i> (genus) <i>Fusobacterium</i> (genus)	Swab and biopsy (5-10)	Pyrosequencing (V1-3)

DPP, days postpartum; DGGE, denaturing gradient gel electrophoresis; qPCR, quantitative polymerase chain reaction.

Porphyromonas, and *Fusobacterium* in metritic cows. Notably, relative abundance of *Bacteroides* appeared to be significantly higher in metritic cows than in healthy cows at 6 ± 2 DPP. Similar findings have been reported in other studies that used different types of samples and techniques [25, 26, 29]. In addition, cows that cured metritis either naturally or with antibiotic treatment displayed a decrease in abundance of *Bacteroides*, *Porphyromonas*, and *Fusobacterium* [24]. Considering all the literature in Table 1, it is reasonable to conclude that *Bacteroides*, *Porphyromonas*, and *Fusobacterium* play a key role in development of metritis. At the same time, it was not clear which bacteria were associated with uterine health due to individual variation of uterine microbiota, but healthy or cured cows appeared to harbor more diverse uterine microbiota [22, 23, 29].

Uterine Pathogens

In metagenomic analysis, *F. necrophorum* was found to be the most abundant species in the uterus, while *P. melaninogenica*, *E. coli*, and *T. pyogenes* were found to be extremely rare (<1% abundance) with no association with metritis [10, 23]. Conversely, *E. coli* was shown to be associated with uterine health, which conflicts with results from previous studies based on culture methods [23]. Herein, we discuss current understanding of uterine bacteria that are known and newly identified in metagenomic sequencing. *P. melaninogenica* that was barely detected in metagenomic sequencing [23] and *T. pyogenes* that is more likely associated with endometritis [30-32] are not described in this paper.

Bacteroides

Bacteroides was the most abundant genus in the uterus of metritic cows and the only genus that was significantly more prevalent in metritic cows than in healthy cows [23]. Also, the relative abundance of *Bacteroides* was shown to be associated with uterine discharge [23], suggesting that *Bacteroides* is the main pathogen causing metritis. In the genus of *Bacteroides*, *B. heparinolyticus* was the most abundant species in the uterus of cows with metritis [23]. However, absolute quantification of this bacterium was not significantly different between healthy and metritic cows [33]. *B. heparinolyticus* was found in the blood and feces of dairy cows, but it was more likely to colonize the uterus [34]. Considering the large proportion of *B. heparinolyticus* in the uterus of healthy and metritic cows, the role of *B. heparinolyticus* in uterine health is warranted. *B. pyogenes* was the second most abundant *Bacteroides* species in the uterus of cows with metritis [23], and it was recently recognized as a

uterine pathogen in dairy cows associated with a fever [10]. *B. pyogenes* was first identified in abscesses and feces of pigs [35], and it has been identified in infected wounds after cat bites in humans [36]. Relatedness of *B. pyogenes* strains isolated from other hosts needs to be investigated to explore the origin and pathogenicity of *B. pyogenes* in the uterus of dairy cows. *Bacteroides* species are capable of causing diseases through a polysaccharide capsule, release of endotoxin (lipopolysaccharides), evasion of the host immune response, production of proteolytic enzymes (e.g., hyaluronidase and chondroitin sulfatase), production of hemolysins, release of enterotoxin (fragolysin), and aerotolerance [37]. Particularly, *Bacteroides* species are known to be resistant to several antibiotics including ceftiofur, which is a widely used antibiotic for treating metritis in dairy cows in the United States and Europe [24, 38].

Porphyromonas

Porphyromonas has recently been considered a uterine pathogen in metagenomic studies [10, 23-25, 29]. Within the genus *Porphyromonas*, *Porphyromonas levii*, *Porphyromonas endodontalis*, and *Porphyromonas somerae* were the most abundant species in the uterus of dairy cows [39]. Among these species, only *P. levii* was determined to be a uterine pathogen by the quantitative PCR analysis [33]. *P. levii* were also known as a pathogen causing bovine foot rot [40] and necrotic vulvovaginitis [41, 42]. It has been demonstrated that *P. levii* has the ability to avoid phagocytosis by polymorphonuclear neutrophils through production of immunoglobulin protease [43]. This may enable them to invade the endometrium during infection [44].

Fusobacterium

Fusobacterium was found to be abundant in both healthy and metritic cows by metagenomic analysis [10, 22, 23]. Nonetheless, it showed the correlation with uterine discharge score and interaction with abundance of *Bacteroides* [23], which implies that *Fusobacterium* is involved in development of metritis. Particularly, *Fusobacterium necrophorum* is a well-known pathogen related to uterine disease in dairy cows, as has been ascertained in culture- [45], PCR- [33], and sequencing-based studies (Table 1). The key virulence factor of *F. necrophorum* includes leukotoxin that is cytotoxic to immune cells specifically in cattle and sheep, and protects them against phagocytosis by neutrophils [46, 47]; despite *F. necrophorum* being well-studied, pathogenicity of bovine uterine isolates has not yet been investigated. *F. necrophorum* appeared to be present with other pathogens such as *T. pyogenes* and *P. levii*, suggesting a synergistic role of causing

uterine disease [44, 45]. Similarly, the coexistence of *Fusobacterium* with *Bacteroides* and *Porphyromonas* was observed in the blood and uterus of dairy cows by network analysis [34]. Therefore, pathogenicity of *F. necrophorum* in the uterus may be attributed to interactions with other bacteria.

Helcococcus

Gram-positive facultative anaerobic *Helcococcus ovis* and *Helcococcus kunzii* were isolated from the uterus of dairy cows with metritis [48, 49]; but their involvement in metritis was not evident. Later metagenomic studies found that *Helcococcus* was associated with metritis [23] and endometritis [20], suggesting it to be a potential uterine pathogen. This is supported in a study by Cunha *et al.* [33] in which *H. ovis* was significantly prevalent in metritic cows than in healthy cows. Because of its presence together with *E. coli* and *T. pyogenes* [48], interaction with other uterine pathogens is likely during metritis development. *H. ovis* was also detected in sheep with subclinical mastitis [50], horses with pulmonary abscess [51] and cattle with valvular endocarditis [52, 53]. However, virulence factors of *H. ovis* remain to be elucidated.

Escherichia coli

Most *E. coli* in the human gastrointestinal tract are commensal microorganisms. However, when they are found in the urinary tract, which is called uropathogenic *E. coli* (UPEC), *E. coli* are highly harmful causing acute or recurrent urinary tract infection in humans [54]. One of the important virulence factors of UPEC is the type 1 pilus tip adhesion, which helps bacteria to bind to human and mouse bladder epithelial cells during urinary tract infection [55]. Likewise, some *E. coli* strains recovered from the uterus of postpartum cows had the *fimH* gene encoding type 1 pilus tip adhesion, and the presence of the *fimH* gene at 1-3 DPP was shown to be associated with metritis [15]. Nevertheless, *E. coli* has turned out to be very rare in the uterus of dairy cows with metritis by metagenomic sequencing analysis [23]; in fact, *E. coli* was shown to be associated with uterine health. The conflicting result may be because of diversity of *E. coli* strains. Indeed, Silva *et al.* [56] observed genomic diversity of *E. coli* strains isolated from the uterus of dairy cows with metritis, and none of the strains showed association with uterine infection. Sheldon *et al.* [57] insisted that there is a particular *E. coli* strain that is more invasive and adherent to endometrial stromal cells than other strains of *E. coli*. To understand pathogenic mechanisms of *E. coli* in the uterus, whole-genome sequencing of *E. coli* strains isolated from the uterus of dairy cows with metritis has been performed [58-60]. Comparative genomic analysis between strains

from healthy and metritic cows is warranted.

Conclusion

In metagenomic sequencing of uterine microbiota, *Bacteroides* and *Fusobacterium* were identified as uterine pathogens; this finding is in agreement with previous findings obtained by culture-dependent methods [23, 29, 61, 62]. *Porphyromonas* and *Helcococcus* have been additionally incriminated as uterine pathogens [20, 23]. Pathogenic bacteria belonging to these genera include *F. necrophorum*, *B. pyogenes*, *P. levii*, and *H. ovis*. Meanwhile, according to deep sequencing and PCR analyses, the role of *E. coli* in metritis development is unclear because they were present in extremely low abundance in the uterus and showed no association with metritis [23, 33]. Nonetheless, it is possible that some *E. coli* are more pathogenic to endometrium of dairy cows, thereby contributing to metritis [57]. For confirmation of association of *E. coli* with metritis, further evidence is warranted.

Illumina MiSeq platform is the preferred methodology in recent metagenomic studies because it provides deep sequencing and a cost-effective method to identify bacterial community. However, 454 pyrosequencing is also a sequencing option for bacterial community studies when long-read sequencing is desired; Illumina MiSeq platform reads a single or paired end up to 300 bp and 454 pyrosequencing platform reads up to 500 bp. The choice of a sequencing platform can be determined based on sample size and purpose of study as both Illumina sequencing and pyrosequencing provide greater sequencing depth and coverage. As seen in Table 1, similar results are derived from recent metagenomic studies regardless of the chosen or selected sequencing platforms.

However, there are some limitations in metagenomic analysis. First, bacterial viability is unclear because metagenomic sequencing analyzes microbial DNA (e.g., 16S rRNA gene) that can be derived from either dead or live bacteria. Also, metagenomic sequencing provides only relative abundance data, and classification of short-reads of DNA is generally accepted up to the genus level. Therefore, culture and PCR methods are still necessary to complement the metagenomic data. Finally, it is possible that uterine microbiota samples can be contaminated during DNA purification as seen in the case of placenta microbiota which showed difficulty distinguishing microbiota from contamination controls [63]. Thus, negative controls with no DNA, such as reagent and water, need to be analyzed with samples to ensure no DNA contamination.

The study of uterine microbiota in dairy cows will improve our knowledge of uterine diseases in women. Uterine

bacteria found in women with puerperal sepsis, pelvic inflammatory disease, or endometrial cancer are similar to those observed in cows with metritis [23, 24, 64-66]. Considering the high prevalence of metritis, availability and accessibility of samples, and the similarity of pathogens that cause uterine disease in cows and humans, we propose that the bovine species is an excellent model by which to explore the role of uterine microbiota in health and disease.

Bacterial community structure is linked to the function of bacterial community. Therefore, metagenomic analysis of uterine microbiota is the first step to understand the role of uterine microbiota in disease and fertility. Currently, we found a specific uterine microbiota associated with metritis, which harbors abundance of *Bacteroides*, *Porphyromonas*, and *Fusobacterium*. These uterine pathogens were also found in the gut and blood of dairy cows, indicating hematogenous transmission of bacteria towards the uterus [34]. This is supported by the fact that bacteremia was detected in more than ~50% of postpartum dairy cows [67], and that uterine pathogens were found in other body sites causing liver abscess [68, 69] and foot rot [40, 70]. Because uterine pathogens were even present in healthy and non-pregnant cows without causing inflammation [71, 72], it is possible that uterine pathogens are normal residents of the bovine uterus, and uterine disease may develop in postpartum cows with high abundance of uterine pathogens by alteration of uterine microbiota. Therefore, modulating uterine microbiota towards limiting abundance of uterine pathogens and improving diversity of uterine microbiota may be necessary for uterine health and fertility.

ORCID: Soo Jin Jeon: <https://orcid.org/0000-0001-7473-3184>; Klíbs N. Galvão: <https://orcid.org/0000-0002-6683-4476>

Authors' contribution

Conceptualization: SJJ
 Data curation: SJJ
 Formal analysis: SJJ
 Writing – original draft: SJJ
 Writing – review & editing: SJJ, KNG

Conflicts of Interest

No potential conflicts of interest relevant to this article was reported.

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