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Quantifying known and emerging uterine pathogens, and evaluating their association with metritis and fever in dairy cows

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ABSTRACT

Metritis is caused by polymicrobial infection; however, recent metagenomic work challenges the importance of known pathogens such as *Escherichia coli* and *Trueperella pyogenes* while identifying potential new pathogens such as *Bacteroides pyogenes, Porphyromonas levii* and *Helcococcus ovis*. This study aims to quantify known and emerging uterine pathogens, and to evaluate their association with metritis and fever in dairy cows. Metritis was diagnosed at 6 ± 2 days postpartum, a uterine swab was collected and rectal temperature was measured. 39 cows were classified into three groups: Healthy (n = 14), Metritis without fever (MNoFever; n = 12), and Metritis with fever (MFever; n = 13). Absolute copy number was determined for total bacteria and for 8 potentially pathogenic bacteria using droplet digital PCR. Both MNoFever and MFever cows had higher copy number of total bacteria, *Fusobacterium necrophorum*, *Prevotella melaninogenica, Bacteroides pyogenes*, *Porphyromonas levii,* and *Helcococcus ovis* than Healthy cows. MNoFever and MFever groups were similar. There was no difference among groups in copy number of *Escherichia coli*, *Trueperella pyogenes*, and *Bacteroides heparinolyticus,* and they all had low copy numbers. Our work confirms the importance of some bacteria identified by culture-based studies in the pathogenesis of metritis such as *Fusobacterium necrophorum* and *Prevotella melaninogenica*; however, it challenges the importance of others such as *Escherichia coli* and *Trueperella pyogenes* at the time of metritis diagnosis. Additionally, *Bacteroides pyogenes*, *Porphyromonas levii,* and *Helcococcus ovis* were recognized as emerging pathogens involved in the etiology of metritis. Furthermore, fever was not associated with the total bacterial load or specific bacteria.

1. Introduction

Metritis is one of the most important and prevalent postpartum diseases in dairy cows, and has a negative effect on fertility, productivity, culling and profitability [1–3], besides affecting animal welfare [4]. The disease is characterized by an abnormally enlarged uterus and a fetid, red-brownish watery uterine discharge within 21 days after calving [5]. The severity of the disease is usually assessed through visual examination of uterine discharge and overall clinical assessment of the cow which includes evaluation of rectal temperature [5]. Of particular interest is the fact that although metritic cows that do not develop a fever have a similar drop in milk yield, they have an increased cure rates [6,7] and improved fertility [6] compared with metritic cows that do develop a fever. The findings of increased cure rates and improved fertility for metritic cows that do not develop a fever indicate that they may have a lower bacterial challenge than metritic cows with a fever. On the other hand, it is also possible that the immune system responds differently to a similar bacterial challenge; therefore, leading to the development of fever in some cows but not in others.

Metritis is widely considered to arise from mixed bacterial infections, primarily by pathogenic gram-negative facultative and obligate anaerobes with the exception of *Trueperella pyogenes*, which is a gram-positive facultative anaerobe [8–10]. Although the bovine uterus is assumed to be sterile before pregnancy, a wide

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range of bacterial species can be found in the uterine lumen of most cows immediately after parturition [10,11]. These opportunistic bacteria are thought to enter the uterus during and immediately after parturition, and their presence in the uterus can cause damage to the endometrium, inflammation, and alter uterine and ovarian function [12,13].

Studies using culture methods have laid the groundwork for our understanding of bacterial uterine disease [13–15]. These culture studies have identified *Escherichia coli* (*E. coli*), *Trueperella* (*Arcanobacterium*) *pyogenes* (*T. pyogenes*), *Fusobacterium necrophorum* (*F. necrophorum*), and *Prevotella melaninogenica* (*P. melanonogenica*) as the main bacteria involved in the pathogenesis of uterine disease [10]. Polymerase chain reaction (PCR) techniques have also been used to identify uterine bacteria, but the targeted bacteria were based on culture studies; therefore, they did not overcome the limitations of culture-based studies [16,17]. Furthermore, PCR was used to identify and not to quantify uterine bacteria. Recent metagenomic studies have provided greater insight into the complex microbiome of the post-partum uterus, and have identified previously uncultured species of bacteria that could be involved in the pathogenesis of metritis. Using 16S rRNA gene sequencing, recent studies observed that species of bacteria from the genera *Bacteroides*, *Fusobacterium*, *Helcoccocus*, and *Porphyromonas* were associated with metritis and potentially fever in dairy cows [11,18]. Furthermore, whereas culture studies report a high prevalence of *E. coli* and *T. pyogenes* [15], 16S rRNA gene sequencing studies report very low prevalence of these pathogens, and instead, report a high prevalence of *Bacteroides spp.*, *Fusobacterium spp.*, *Porphyromonas spp.*, and *Helcococcus spp.* [11,18,19]. Although 16S rRNA gene sequencing is considered to be very accurate up to genus level, species data need to be confirmed with more specific techniques such as PCR because of the potential for species misassignment due to the short reads generated by 16S rRNA gene sequencing [20]. Another advantage of PCR is that it can generate absolute quantification of bacteria; whereas 16S rRNA sequencing only provides relative abundance data. Although real-rime quantitative PCR (qPCR) can be used for bacterial quantification, the need for development of standard curves is a limiting factor when having to assess bacteria that have not been cultured or cannot be easily cultured, or that whole genome sequences are not available. Droplet digital PCR is a system that works by partitioning PCR samples into ~1 nL individual reactions and estimating copy number based on the ratio of positive to negative reactions, which eliminates the need for a standard curve [21,22]. Furthermore, although qPCR and ddPCR have been shown to have good correlation, ddPCR was found to be more accurate because of greater precision (lower coefficient of variation) and greater reproducibility [21,22], making ddPCR an ideal method for bacterial absolute quantification. Therefore, the objective of this study was to quantify known bacterial species identified from culture studies and bacterial species identified in a previous study using 16S rRNA gene sequencing, and evaluate their association with the development of metritis and fever in lactating dairy cows. From culture studies we targeted *F. necrophorum*, *T. pyogenes*, *E. coli*, and *P. melaninogenica* [15]. From our previous 16S rRNA gene sequencing study, we targeted *Bacteroides pyogenes* (*B. pyogenes*), *Bacteroides heparinolyticus* (*B. heparinolyticus*), *Fusobacterium gonidiaformans* (*F. gonidiaformans*), *Porphyromonas levii* (*P. levii*), and *Helcococcus ovis* (*H. ovis*) [11,18]. Finally, a consensus sequence based on an alignment of 962,279 bacterial 16S rRNA sequences was targeted to assess overall bacterial count [23].

With guidance from relative abundance data generated by 16S rRNA gene sequencing, absolute quantification methods can be used to verify and more accurately assess individual bacterial species quantities and its association with disease.

2. Materials and methods

2.1. Facilities and animals

All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee (no. 201207405 and no. 201408598). A total of 125 Holstein cows from two dairy farms (92 from dairy A and 33 from dairy B) located in north central Florida were used for this study. All animals sampled were lactating Holstein Friesian cows enrolled in the study at calving.

2.2. Uterine swab collection and metritis diagnosis

All cows had a uterine swab collected, and had the uterine discharge evaluated at 4, 6, and 8 days postpartum (6 ± 2 days postpartum). For swab collection, the perineum area of the cow was cleaned and disinfected with 70% ethyl alcohol, the vulva was spread open and a double-guarded sterile swab (McCullough double-guarded uterine culture swab; Jorgensen Labs Inc., Loveland, CO) was introduced in the vagina, passed into the cranial portion of the vagina, and then manipulated through the cervix. At the uterine body, the internal plastic sheath was pushed through the protecting cap and then the swab was exposed and rolled against the uterine wall. Successful passage of the device through the cervix was aided and confirmed by palpation per rectum. The swab was then retracted into the internal plastic sheath, which was then retracted into the external plastic sheath and removed from the cows. Once removed, the internal plastic sheath containing the swab was capped and stored in ice for transport to the laboratory. In the laboratory, swab samples were suspended in 0.5 mL lysogeny broth and stored at −80 °C.

Uterine discharge was evaluated for diagnosis of metritis after uterine swab collection using a metricheck device (Metricheck, Simcro, New Zealand). Uterine discharge scoring method on a 5 point scale: 1 = not fetid normal lochia, viscous, clear, red, or brown; 2 = cloudy mucoid discharge with flecks of pus; 3 = not fetid mucopurulent discharge with <50% pus; 4 = not fetid mucopurulent discharge with >50% pus; 5 = fetid red-brownish, watery discharge was used as previously described [18]. Cows with a uterine discharge score of 5 were diagnosed as having metritis. Rectal temperature was measured using GLA M750 Digital Thermometer for Large Animals. Fever was defined as a rectal temperature ≥39.5 °C. Cows were classified as metritis with a fever if they had a uterine discharge score of 5 and had rectal temperatures of ≥39.5 °C at the time of metritis diagnosis. Cows were classified as metritis without a fever if they had a uterine discharge score of 5 and had rectal temperatures of <39.5 °C at the time of metritis diagnosis. Cows with uterine discharge scores <5 were classified as healthy.

2.3. Inclusion and exclusion criteria

Initially, of the 125 cows, 65 were classified as healthy, 26 were classified as metritis with a fever and 34 were classified as metritis without a fever. However, to be included in the study, cows with metritis without a fever and healthy cows could not have had a fever at any time during the observation period. Cows with metritis and a fever could have had a fever before or after the diagnosis of metritis in addition to having a fever at the time of diagnosis of metritis. Healthy cows that developed any other disease during the observation period were excluded from the study. Cows that received antibiotic treatments prior to sample collection were excluded from the study. After exclusions, a total of 39 swab samples were available for PCR analysis; 13 from cows that developed