Veterinary Microbiology 214 (2018) 132–139

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Shift of uterine microbiota associated with antibiotic treatment and cure of metritis in dairy cows

Soo Jin Jeon[a], Fabio S. Lima[b,**], Achilles Vieira-Neto[a,c], Vinicius S. Machado[d], Svetlana F. Lima[d], Rodrigo C. Bicalho[d], Jose Eduardo P. Santos[a,c], Klibs N. Galvão[a,c,*]

[a] Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL, USA
[b] Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois, Urbana-Champaign, IL, USA
[c] D. H. Barron Reproductive and Perinatal Biology Research Program, University of Florida, Gainesville, FL, USA
[d] Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA

ARTICLE INFO

Keywords:
Uterus
Microbiota
Metritis
Ceftiofur
Ampicillin
16S metagenomic sequencing
Cow

ABSTRACT

Broad-spectrum antibiotics such as ceftiofur and ampicillin are recommended for the treatment of metritis in dairy cows. Nonetheless, little is known about the impacts of antibiotics on the uterine microbiota. Here, we evaluated the shift in uterine microbiota after treating metritic cows with ceftiofur or ampicillin, and also gained insight into the uterine microbiota associated with cure of metritis. Uterine swabs from ceftiofur-treated, ampicillin-treated, and untreated metritic Holstein cows were collected on the day of metritis diagnosis (D1) and on D6 and then used for genomic DNA extraction and sequencing of the V4 hypervariable region of the 16S rRNA gene on the Illumina MiSeq platform. The uterine microbiota consolidated over time by decreasing species richness and increasing evenness; therefore, becoming more homogeneous. The uterine microbial community showed distinct clustering patterns on D6 according to antibiotic treatment, which could be attributed to more dynamic changes in the microbial structure from D1 to D6 in ceftiofur-treated cows. Ceftiofur led to significant changes at the community level, phylum level, and genus level, whereas the changes in ampicillin and untreated cows, although following the same pattern, were mostly non-significant. Bacteroidetes was significantly increased in ceftiofur-treated cows but was not changed after ampicillin and no treatment. Different responses to antibiotics were observed in *Porphyromonas*, which increased in relative abundance with ceftiofur and decreased with ampicillin. Regardless of treatment group, failure to cure metritis was associated with a decrease in diversity of uterine microbiota and an increase in the relative abundance of *Bacteroides*, *Porphyromonas*, and *Fusobacterium*.

1. Introduction

Metritis, which is an acute inflammation of the uterine wall, is one of the most common and costly diseases in postpartum dairy cows (Sheldon et al., 2006; Galvão and Santos, 2014). Metritis is caused by polymicrobial infection with Gram-negative (*Bacteroides, Porphyromonas, and Fusobacterium*) and Gram-positive bacteria (*Trueperella*, *Peptostreptococcus,* and *Helcococcus*) (Santos et al., 2011; Peng et al., 2013; Locatelli et al., 2013; Jeon et al., 2015; Knudsen et al., 2016; Jeon et al., 2016b). Because of the polymicrobial nature of metritis, broad-spectrum antibiotics are recommended for treatment (Drillich et al., 2001; Smith and Risco, 2002; Lima et al., 2014; Haimerl et al., 2017). We recently reported that two major phyla, Bacteroidetes and Fusobacteria, dominate the microbiome of cows with metritis (Jeon et al., 2015); therefore, antibiotic effectiveness against these two phyla may be critical for metritis cure.

Ceftiofur is a broad-spectrum cephalosporin that shows strong *in vitro* antimicrobial activity against uterine pathogens (Drillich et al., 2001; Sheldon et al., 2004; Malinowski et al., 2011), except for Bacteroidetes (Samitz et al., 1996), in which presence of beta-lactamase genes confer resistance to most beta-lactam antibiotics (Uraz and Turkmen, 1999). Ceftiofur is usually the preferred option for treatment of metritis in cows because it does not require milk withdrawal. However, the use of ceftiofur has been associated with the development and dissemination of resistance to ceftriaxone, a third-generation cephalosporin only available for humans (Tragesser et al., 2006). Thus, the sustained use of ceftiofur in food animals raises concerns about the emergence of antibiotic resistant bacteria in agriculture, which can then

* Corresponding author at: Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL, USA.
** Corresponding author at: Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois, Urbana-Champaign, IL, USA.
E-mail addresses: flima@illinois.edu (F.S. Lima), Galvãok@ufl.edu (K.N. Galvão).

https://doi.org/10.1016/j.vetmic.2017.12.022
Received 16 November 2017; Received in revised form 27 December 2017; Accepted 27 December 2017

affect humans (Tragesser et al., 2006; Liu et al., 2016).

Alternatives to decrease the use of ceftiofur could include the use of other classes of drugs approved for use in lactating dairy cattle such as tetracyclines or the use of older beta-lactams. Ampicillin is a broad-spectrum penicillin that is approved for use in lactating dairy cows that could be alternatively used for the treatment of metritis in cows. Although penicillins are structurally related to cephalosporins in that they all contain a beta-lactam ring, they are different enough so that penicillinases cannot hydrolyze cephalosporins efficiently (Hall and Barlow, 2004; Shahid et al., 2009). The evolution of beta-lactamases with increased activity towards cephalosporins seriously threaten the utility of these antibiotics (Hall and Barlow, 2004).

Administration of ampicillin trihydrate has been shown to result in therapeutic concentrations in uterine tissues and lochia (Credille et al., 2015). In a recent study, metritic cows treated with ampicillin were found to have a quicker recovery than cows treated with ceftiofur, in which the cure rate of metritis was greater for ampicillin than for ceftiofur 7 days after the first treatment, although both antibiotics became comparable 12 days after the first treatment (Lima et al., 2014). Nonetheless, it is not clear why ampicillin-treated cows had a faster recovery than ceftiofur because *in vitro* activity against major uterine pathogens such as Bacteroidetes and Fusobacteria (Jeon et al., 2015) have been shown to be higher for third-generation cephalosporins than for ampicillin (Johnson, 1993). The inflammation associated with metritis has been shown to decrease tissue concentrations of ceftiofur derivatives (von Krueger et al., 2013); therefore, it is possible that ampicillin is better able to reach minimum inhibitory concentrations against Bacteroidetes and Fusobacteria in the uterus than ceftiofur.

The use of next-generation sequencing provides a valuable tool to understand the shift in the microbiome structure in the uterus (Jeon et al., 2015), and could be used to better understand the difference in outcome (i.e. cure or no cure) after treatment of metritis with ceftiofur or ampicillin. In this study, we performed 16S metagenomic sequencing of uterine swabs collected from 44 dairy cows with metritis that were left untreated or were treated with ceftiofur or ampicillin. We sought to explore uterine microbial shifts associated with antibiotic treatment, and to identify uterine microbiota associated with the cure of metritis.

2. Materials and methods

2.1. Ethics approval

All animal procedures were approved by the University of Florida Institute of Food and Agricultural Sciences Animal Research Committee (Protocol number 013-12ANS), and all experiments were performed in accordance with relevant guidelines and regulations.

2.2. Experimental design

A total of 44 Holstein dairy cows diagnosed with metritis from a commercial dairy in Central Florida milking 5000 cows were enrolled in this study. Metritis was diagnosed by the presence of fetid, red-brownish, watery uterine discharge regardless of fever, as previously reported (Lima et al., 2014; Jeon et al., 2016b). Metritic cows were randomly assigned to one of three groups: CEF (n = 15) received 2.2 mg/kg of ceftiofur i.m. for five days (Excenel® RTU sterile suspension, Zoetis, Madison, NJ, USA); AMP (n = 15) received 11 mg/kg of ampicillin trihydrate i.m. for five days (Polyflex®, Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO, USA); UNT (n = 14) received no treatment upon metritis diagnosis. The random number formula (RANDBETWEEN) in Excel® was used to generate the order of treatments, and hence achieve randomization of treatment assignment. Sample size was based on previous studies that evaluated shifts in the uterine microbiota in cows that developed metritis (Santos and Bicalho, 2012; Jeon et al., 2015). Those studies used 8 to 12 cows per group. Uterine swab samples were collected prior to antibiotic treatment on the day of metritis diagnosis (D1) right before the first antibiotic treatment in antibiotic-treated cows and 5 days after metritis diagnosis (D6), which was one day after the last antibiotic treatment in antibiotic-treated cows. Cure of metritis was determined two days after the last treatment (D7), and was characterized by absence of fetid red-brownish watery uterine discharge. A diagram illustrating the experimental design is depicted in Supplementary Fig. S1.

2.3. Sampling, DNA extraction, and sequencing

Uterine swab samples were collected from 44 cows on D1 and D6 using a 30" double-guarded sterile culture swab (Continental Plastics Corporation, Delavan, WI). Briefly, the instrument was gently passed through the cervix and positioned in the uterine body where the internal sheath and the swab were exposed, and the swab was gently rolled against the uterine wall. The swab was retracted within the double sheath before removal from the cow. The swab was stored in a 15 ml conical sterile tube and placed on ice until return to the laboratory where it was stored at −80 °C until DNA extraction.

Swab samples were thawed and homogenized in 2 ml of sterile phosphate-buffered saline. The swabs were then removed, and the solution was transferred to a 2 ml tube and centrifuged at 13,200*g* for 10 min. The supernatant was discarded and the remaining pellet was resuspended in 400 ml of nuclease-free water and incubated at 56 °C for 12 h with 40 µl of proteinase K (IBI Scientific, Peosta, IA, USA), 180 µl of tissue lysis buffer, and 40 µl of lysozyme (QIAamp DNA Mini kit, Qiagen, Valencia, CA, USA) to maximize bacterial DNA extraction. Isolation of genomic DNA was performed by using a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. DNA concentration and purity were evaluated by optical density using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) at the wavelengths of 230, 260, and 280 nm, and the OD260/280 ratio of DNA ranged between 1.7 and 2.0. The 16S rRNA genes were amplified by PCR from individual metagenomic DNA samples using barcoded primers. For amplification of the V4 hypervariable region of the bacterial 16S rRNA gene, primers 515F and 806R were used according to a previously described method (Caporaso et al., 2012) optimized for the Illumina MiSeq platform. The 12-bp error-correcting Golay barcodes were used to tag the 16S rRNA genes as described previously (Caporaso et al., 2012). The 5′-barcoded amplicons were generated in triplicate using 12 to 300 ng DNA template (isolated from uterine samples), 1 X GoTaq Green Master Mix (Promega, Madison, WI), 1 mM $MgCl_2$, and 10 mM each primer. The PCR conditions for the 16S rRNA gene consisted of an initial denaturing step of 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 1 min, and 72 °C for 90 s, and a final elongation step of 72 °C for 10 min. Replicate amplicons were pooled and purified with a QIAquick PCR purification kit (Qiagen), and visualized by electrophoresis through 1.2% (wt/vol) agarose gels stained with 0.5 mg/ml ethidium bromide before sequencing. Reactions with blank controls, in which no DNA was added to the reaction mixture, were performed. In all cases these blank controls failed to produce visible PCR products; these samples were not analyzed further. Purified amplicon DNA was quantified using the Quant-iT PicoGreen double-stranded DNA (dsDNA) assay kit (Life Technologies Corporation, Carlsbad, CA, USA). Amplicon aliquots were standardized to the same concentration and then pooled into one of one run according to individual barcode primers of the 16S rRNA gene. Final equimolar libraries were sequenced using the MiSeq reagent kit V2 for 300 cycles (read length of 300 bases, sequenced as single read) on the MiSeq platform (Illumina Inc., San Diego, CA, USA). The sequencing primers target sequencing of the 533–786 region (V4 region) of the 16S rRNA gene 6 in the Escherichia coli strain 83972 sequence (greengenes accession no. prokMSA_id:470367) (Caporaso et al., 2012); therefore, 300 bases would cover the whole length of the targeted region.

The 16S rRNA gene sequences obtained from the MiSeq platform