Minimum inhibitory concentrations of frequently used antibiotics against *Escherichia coli* and *Trueperella pyogenes* isolated from uteri of postpartum dairy cows

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ABSTRACT

The objective of this study was to determine minimum inhibitory concentrations (MIC) of frequently used antimicrobials for *Escherichia coli* and *Trueperella pyogenes* isolated from postpartum bovine uteri of cows with acute puerperal metritis (APM, $n = 67$), cows suspected to have APM ($n = 37$), and healthy cows ($n = 37$) and to evaluate possible differences in MIC according to clinical signs. Cows with APM had reddish-brown, fetid vaginal discharge and rectal temperature (RT) $\geq 39.5^{\circ}\text{C}$ within 21 d in milk; cows suspected to have APM had either reddish-brown, fetid vaginal discharge or RT $\geq 39.5^{\circ}\text{C}$ within 21 d in milk; and healthy cows had neither fetid discharge nor RT $\geq 39.5^{\circ}\text{C}$. Samples were collected from cows on commercial dairy herds ($n = 7$) using the cytobrush technique. A total of 37 *T. pyogenes* isolates and 85 *E. coli* isolates were tested. Ceftiofur, a third-generation cephalosporin that is often used to treat APM, was the focus of analysis. *Trueperella pyogenes* and *E. coli* were isolated more often from samples of cows with APM (46 and 90%, respectively) compared with samples from healthy cows (19 and 54%, respectively). Regarding cows suspected to have APM, *T. pyogenes* and *E. coli* were numerically more often isolated (30 and 70%, respectively) than in healthy cows (19 and 54%, respectively). Minimum inhibitory concentrations of ceftiofur were low. For *T. pyogenes* and *E. coli*, MIC₅₀ (concentration that inhibited growth of 50% of isolates) were 0.25 and 0.5 $\mu\text{g/mL}$ and MIC₉₀ (concentration that inhibited growth of 90% of isolates) were 0.5 and 1 $\mu\text{g/mL}$, respectively. Although ceftiofur inhibited all *T. pyogenes* at the highest concentration tested (64 $\mu\text{g/mL}$), the growth of 5.9% of *E. coli* was not impaired. Recently, ampicillin has been suggested as an alternative treatment for

APM. Although the *T. pyogenes* isolates exhibited low MIC in general (MIC₅₀ ≤ 0.015 $\mu\text{g/mL}$ and MIC₉₀ = 0.06 $\mu\text{g/mL}$) and 81.1% of all *T. pyogenes* could be inhibited at the lowest ampicillin concentration tested, 11.8% of the *E. coli* isolates were not impaired at the highest concentration (64 $\mu\text{g/mL}$) tested in this study. The MIC₅₀ and MIC₉₀ of *E. coli* were 4 and ≥ 128 $\mu\text{g/mL}$, respectively. We detected no difference in the MIC distributions of ceftiofur or ampicillin among isolates from the 3 APM groups. In summary, *E. coli* with high MIC against ceftiofur as well as against ampicillin were found in this study.

Key words: minimum inhibitory concentration, antimicrobial susceptibility, antibiotic, metritis

INTRODUCTION

Infectious uterine diseases are common in dairy cows, with an incidence of acute puerperal metritis (APM) between 14.8 and 40.9% (Chapinal et al., 2011; Sannmann et al., 2013; Lima et al., 2014). Acute puerperal metritis is characterized by reddish-brown, fetid, watery vaginal discharge and rectal temperature (RT) $\geq 39.5^{\circ}\text{C}$ within the first 21 DIM, and it leads to pain response detectable during transrectal palpation (Sheldon et al., 2006; Stojkov et al., 2015). Different aerobic and anaerobic bacteria can be isolated from the infected uterus, including *Escherichia coli*, *Trueperella pyogenes*, *Prevotella* spp., *Fusobacterium necrophorum*, *Fusobacterium nucleatum*, *Mannheimia haemolytica*, *Staphylococcus* spp., and *Streptococcus* spp. (Sheldon et al., 2004; Williams et al., 2005; Santos et al., 2011). *Trueperella pyogenes* and *E. coli* are known to cause endometrial lesions and therefore are considered the major pathogens associated with APM (Sheldon et al., 2004; Santos et al., 2010b; de Boer et al., 2015). Because of the wide range of potential pathogens, antimicrobials with broad-spectrum activity are primarily indicated, and APM is commonly treated with ceftiofur, a third-generation cephalosporin (Lima

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et al., 2014; Machado et al., 2014). The reason for the widespread use of ceftiofur administered parenterally is its high efficacy (Drillich et al., 2001; Chenault et al., 2004). Moreover, there is no withdrawal time for milk because the amounts of residue appearing in milk are below the tolerance for human consumption (Scientific Advisory Group on Antimicrobials of the Committee for Medicinal Products for Veterinary Use, 2009; Lima et al., 2014). Third-generation cephalosporins, however, are characterized as critically important antimicrobials for human and veterinary medicine (World Health Organization, 2013; World Organization for Animal Health, 2015) and their use in food-producing animals could lead to an increased prevalence of resistance (Scientific Advisory Group on Antimicrobials of the Committee for Medicinal Products for Veterinary Use, 2009). As antibiotic resistance is recognized as a top public health challenge of the 21st century (Thomson et al., 2004; Centers for Disease Control, 2015), veterinarians should support the prudent use of antimicrobials as a risk management option to reduce the emergence and spreading of resistances.

When deciding on antimicrobial treatment, susceptibility testing of pathogens is advisable to select an appropriate antimicrobial (Sheldon et al., 2004; Pyorala et al., 2014). Minimum inhibitory concentrations can be determined by the broth dilution method for various pathogens. This methodology is also suitable for monitoring resistance over time (Cortinhas et al., 2013). The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) provide clinical breakpoints of MIC for different veterinary and human pathogens and antimicrobials. Clinical breakpoints are determined by a review of MIC distribution data, pharmacokinetic-pharmacodynamic parameters, and clinical response and should be used in routine clinical laboratory work to inform the choice of therapy in the patient (Silleby, 2012; EUCAST 2013). The CLSI defines an isolate as susceptible when the infection may be appropriately treated with the dosage regimen of an antimicrobial agent recommended for that type of infection and infecting species. No MIC clinical breakpoints to classify pathogens into susceptible, intermediate, or resistant, however, have been established for bovine uterus-borne *E. coli* or *T. pyogenes* (de Boer et al., 2015). Application of clinical breakpoints of other host species, pathogens, antimicrobial agents, or diseases (e.g., human breakpoints or breakpoints for bovine mastitis) as interpretive criteria is questionable (Werckenthin et al., 2007), and in vitro susceptibility of a specific pathogen does not guarantee clinical outcome (Giguère and Tessmann, 2011). To reduce confusion in terminology, EUCAST introduced the definition of

epidemiological cut-off values in contrast to clinical breakpoints. Epidemiological cut-off values are determined exclusively by the distribution of MIC values, are independent of dosing regimens, and are used to detect development of microbiological resistance to antimicrobial agents (Dalhoff et al., 2009; EUCAST, 2013). This illustrates the need of comparable MIC data for bovine uterine pathogens for future development of clinical breakpoints as well as epidemiological cut-off values for rational therapy and monitoring of MIC values over time.

In 2002 and 2003, ceftiofur hydrochloride (Excenel RTU, Zoetis, Parsippany, NJ) was initially approved for treatment of APM in the United States (US FDA, 2002) and Germany (iMedikament, 2003). Only a few studies have evaluated MIC for pathogens originating from the bovine uterus; therefore, limited data on in vitro susceptibilities or the development of resistance of pathogens against ceftiofur are available (Sheldon et al., 2004; Santos et al., 2010a,b; de Boer et al., 2015). Moreover, little is known about the relation between MIC and clinical signs of disease.

Therefore, the main objective of this study was to assess MIC of commonly used antibiotics for the major APM-causing bacteria *E. coli* and *T. pyogenes* isolated from postpartum bovine uteri of cows housed on 7 farms in Germany. Moreover, MIC according to clinical symptoms of APM were evaluated.

MATERIALS AND METHODS

Farms and Cows

Samples were collected from a convenience sample of dairy cows with median DIM of 6 (interquartile range: 4 to 7) in 7 commercial farms in northeastern Germany from June 2013 to February 2015 as part of a previously published study on the treatment of APM (Pohl et al., 2016). Body temperature and vaginal discharge of early lactation cows were evaluated daily by trained farm personnel. Farms were visited every other week and diagnoses were confirmed before samples of cows with APM (i.e., 2 clinical signs of APM: reddish-brown, fetid vaginal discharge and RT $\geq 39.5^{\circ}\text{C}$ within 21 DIM, $n = 67$); cows suspected to have APM (i.e., 1 clinical sign of APM: reddish-brown, fetid vaginal discharge or RT $\geq 39.5^{\circ}\text{C}$ within 21 DIM, $n = 37$); and healthy cows (i.e., no clinical signs of APM: no discharge or non-fetid lochia and RT $< 39.5^{\circ}\text{C}$, $n = 37$) were taken.

Participating farms were recruited based on convenience. Herd size ranged from 732 to 2,244 with 677 to 1,319 milking cows. Cows were managed according to the guidelines set by the International Cooperation on Harmonisation of Technical Requirements for Regis-

tration of Veterinary Medical Products (Hellmann and Radeloff, 2000). The experimental procedures reported herein were conducted with the approval of the Institutional Animal Care and Use Committee of Freie Universität Berlin (Germany). Cows were housed indoors in freestall barns with different beddings, (e.g., rubber mats, straw, or dried fermentation product from the on-site biogas plant) and were fed TMR. The TMR fed to prepartum and to early lactation cows were formulated to meet or exceed minimum nutritional requirements for high-producing dairy cows (NRC, 2001). Reproductive performance and medical treatments were recorded and stored by using the herd management software (Herde, version 5.81, dsp Agrosoft GmbH, Ketzin, Germany).

An a priori sample size calculation was not possible, because the prevalence of *E. coli* and *T. pyogenes* and the MIC distributions were unknown for dairy cows in Germany.

Sample Collection

Sample collection was performed by one veterinarian (A. Pohl) throughout the study. Uterine bacteriological swab samples were collected from the uterine body of cows using the cytobrush technique previously described (Sens and Heuwieser, 2013). In brief, the brush was double-guarded by 2 plastic sheaths that prevented bacterial contamination of the brush with pathogens of origin other than the uterus. The vulva was first dry cleaned with cellulose wipes, and then the cytobrush (CytologyBrush for mares, Minitüb GmbH, Tiefenbach, Germany) was inserted into the vagina and passed through the cervix into the uterus. The internal sheath was then pushed through the rubber cap of the external sheath and the brush was pushed forward and brought into contact with uterine fluids. The brush was then retracted into its sheath and gently pulled out of the uterus. Thereafter, the brush was inserted into transport medium, which contained a balanced formula of reducing agents and resazurin in a buffered isotonic agar base (BBL Port-A-Cul, BD Diagnostics/Diagnostic Systems, Heidelberg, Germany). The sample was then transported to the laboratory (Institute of Microbiology and Epizootics, Faculty of Veterinary Medicine, Freie Universität, Berlin, Germany).

Microbiology

A total of 141 cytobrush samples were analyzed by aerobic and anaerobic culture after direct inoculation of the cytobrush on suitable agar plates and after enrichment in medium and subsequent culture on agar (all agar purchased from Oxoid, Wesel, Germany). For the detection of aerobic bacteria, Columbia blood agar

(5% sheep blood), Gassner agar, and Brilliance UTI Clarity agar were inoculated with the cytobrush within 12 h after sampling for 24 to 48 h (aerobic, 36°C). For the evaluation of the presence of obligate anaerobic bacteria, inoculation was carried out on Columbia blood agar (5% sheep blood) with added L-cysteine (Merck, Darmstadt, Germany), hemin (Sigma-Aldrich, Hamburg, Germany), vitamin K1 (Roche, Mannheim, Germany), and lysed sheep blood 0.5% (Oxoid) and on an additional plate with the same agar plus gentamicin (Hexal, Holzkirchen, Germany), for 48 to 72 h (anaerobic, 36°C). Chocolate agar was used for cultivation of fastidious bacteria for 24 to 48 h (36°C, microaerobic, 7% CO₂). In addition, an enrichment culture with brain-heart infusion broth (Oxoid) was inoculated from each cytobrush at 37°C for 24 h, which was transferred to Columbia blood agar (5% sheep blood). Identification of species was carried out by Gram stain, oxidase and catalase tests according to standard operating protocols (Versalovic et al., 2011), and API strips (bioMérieux, Nürtingen, Germany) and verified with matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) MS (Bruker Daltonik, Bremen, Germany).

After identification, isolates were transferred to brain-heart infusion broth and incubated at 37°C until turbidity (indicating bacterial growth) was visible approximately 4 h later. Eight hundred microliters of the broth was then mixed with 200 µL of glycerol and stored at -70°C until used.

MIC Determination

The pathogens for determination of MIC used in this study included 1 isolate from each cow of which *E. coli*, *T. pyogenes*, or both had been isolated. In 16 samples, 2 or 3 phenotypically different strains of *E. coli* were present (according to morphology or hemolysis); in these cases, more than one isolate per cow was analyzed for MIC. The inclusion criterion of isolates for MIC determination was no local or systemic antimicrobial treatment after calving and before sampling. Thus, 12 *T. pyogenes* and 21 *E. coli* were discarded because inspection of the records found that the cows had been treated before sampling. Seventy-four isolates of metritic cows, 38 isolates of cows suspected to have APM, and 10 isolates of healthy cows were used for analysis. A total of 37 *T. pyogenes* and 85 *E. coli* isolates were evaluated for determination of MIC.

The broth microdilution method to determine MIC for the *E. coli* isolates was carried out in accordance with CLSI guidelines (CLSI, 2013). The MIC for *T. pyogenes* was determined using broth microdilution according to recently described protocols (Werckenthin et al., 2007; de Boer et al., 2015). For this, custom-made

microtiter plates (produced by MCS Diagnostics based on Sensititre, Trek Diagnostic Systems Ltd., Swalmen, the Netherlands) were used. The test ranges for each antimicrobial agent are shown in Table 2 for *T. pyogenes* and Table 3 for *E. coli*. The test media were cation-adjusted Mueller-Hinton broth (*E. coli*) and cation-adjusted Mueller-Hinton broth supplemented with 2% (vol/vol) lysed horse blood (*T. pyogenes*). The microtiter plates were incubated at $35 \pm 2^\circ\text{C}$ in ambient air (*E. coli*) or in a CO_2 -enriched environment (*T. pyogenes*) and the results were read after 24 h (*E. coli*) or 48 h (*T. pyogenes*). *Trueperella pyogenes* ATCC 19411 and *E. coli* ATCC 25922 were used as quality control strains and were tested 4 and 5 times together with MIC determination. All susceptibility results from quality control strains were within the prescribed ranges.

Statistical Analysis

The MIC was defined as the lowest concentration of an antimicrobial that inhibited any visible growth of an isolate (Saini et al., 2013). The concentrations that inhibited the growth of 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates was calculated for each antimicrobial (de Boer et al., 2015). Data were analyzed using SPSS for Windows (version 22, SPSS Inc., Munich, Germany) and Medcalc (version 15.0., Mariakerke, Belgium). A binary logistic regression model was used to evaluate the effect of signs indicative of APM (2 signs, 1 sign, no signs) on the occurrence of pathogens. Models were built according to the model-building strategies provided by Dohoo et al. (2009), and cow within farm was included as random effect. Odds ratios for occurrence of pathogens in cows with 2 or 1 signs compared with cows with no signs of APM (reference category) and 95% confidence intervals were calculated. The effect of signs of APM on MIC of ceftiofur and ampicillin were analyzed in a generalized linear mixed model ANOVA. Minimum inhibitory concentrations were previously log-transformed to achieve a normal distribution of values. Models were, again, built according to the model-building strategies provided by Dohoo et al. (2009) and cow within farm was included as random effect.

RESULTS

A total of 141 samples were collected from 67 cows with APM, 37 cows suspected to have APM, and 37 healthy cows. Distribution of isolated pathogens considering the clinical signs indicative of APM is given in Table 1. No bacterial growth occurred in 5 samples from healthy cows and in 1 sample of a cow suspected to have APM. Cows with 2 signs of APM had 7.16

(95% CI: 2.58–19.84; $P < 0.001$) times the odds of having *E. coli* compared with healthy cows and 3.69 (95% CI: 1.4–9.73; $P = 0.009$) times the odds of having *T. pyogenes* compared with healthy cows. Compared with healthy cows, cows with 1 or 2 signs of APM had 6.54 (95% CI: 1.71–25.06; $P = 0.006$) or 10.42 (95% CI: 2.95–36.73; $P < 0.001$) times the odds of having *Fusobacterium* spp. Moreover, cows with 2 signs of APM had 2.94 (95% CI: 1.15–7.50; $P = 0.024$) times the odds of having *Prevotella* spp. compared with healthy cows. Cows with 2 signs of APM also had 4.19 (95% CI: 1.52–11.56; $P = 0.006$) times the odds of having *Bacteroides fragilis* compared with healthy cows.

For determination of MIC, 37 *T. pyogenes* and 85 *E. coli* from 120 cows were evaluated according to the inclusion criteria described above. Twenty-nine (24%) and 93 (76%) isolates were obtained from primiparous and multiparous cows, respectively. Frequency distributions of MIC for a range of antimicrobials against *T. pyogenes* and *E. coli* are given in Tables 2 and 3.

For *T. pyogenes*, the MIC_{50} of ceftiofur was 0.25 $\mu\text{g/mL}$ and the MIC_{90} was 0.5 $\mu\text{g/mL}$. For *E. coli*, the MIC_{50} of ceftiofur was 0.5 $\mu\text{g/mL}$ and the MIC_{90} was 1 $\mu\text{g/mL}$. Variations in distribution of MIC values of ceftiofur for *E. coli* and *T. pyogenes* among the 3 study groups are given in Table 4. No differences were found between logMIC of ceftiofur of cows with 2 or 1 signs of APM compared with no signs of APM for *T. pyogenes* or *E. coli* ($P = 0.321$ or $P = 0.515$). From all *E. coli* recovered from cows with 2 signs of APM, however, 9.8% were not inhibited at the highest concentration of ceftiofur available. For ampicillin, no differences could be found between logMIC of cows with 2 or 1 signs of APM compared with no signs of APM for *T. pyogenes* or *E. coli* ($P = 0.117$ or $P = 0.647$).

The cumulative distribution of MIC of ceftiofur that inhibit the growth of *E. coli* and *T. pyogenes* is shown in Figure 1. A summary of MIC values of ceftiofur against *T. pyogenes* and *E. coli* from our study and 4 other studies is given in Table 5. The MIC_{50} and MIC_{90} of *T. pyogenes* varied by 6 (<0.06 to 2 $\mu\text{g/mL}$) and 9 (0.125 to 32 $\mu\text{g/mL}$) doubling dilutions among studies. The MIC_{50} and MIC_{90} of *E. coli* differed by 3 (0.25 to 1 $\mu\text{g/mL}$) and 2 (0.5 to 1 $\mu\text{g/mL}$) doubling dilutions among studies. Three of the studies provided data deriving only from a single farm in the United Kingdom (Sheldon et al., 2004) or the United States (Santos et al., 2010a,b). Samples from another study were collected from pasture-based cows from 7 herds in New Zealand (de Boer et al., 2015). Regarding the latter, differences in bacterial species and strains are likely in contrast to confinement housing, as has been demonstrated for mastitis (McDougall, 2002; Parker et al., 2008; Pyorala and Taponen, 2009).

Table 1. Frequency distribution [% of cows (no./total no.)] with clinical signs indicative of acute puerperal metritis (2 signs, 1 sign, no sign) and isolated bacterial species isolated from 141 samples

Isolated pathogen	No. of clinical signs of metritis ¹			<i>P</i> -value ²	Total
	2	1	0		
<i>Escherichia coli</i>	90 (60/67) ^b	70 (26/37) ^a	54 (20/37) ^a	0.001	75 (106/141)
<i>Trueperella pyogenes</i>	46 (31/67) ^b	30 (11/37) ^{ab}	19 (7/37) ^a	0.021	35 (49/141)
<i>Streptococcus uberis</i>	66 (44/67)	68 (25/37)	54 (20/37)	0.419	63 (89/141)
<i>Streptococcus dysgalactiae</i>	3 (2/67)	3 (1/37)	3 (1/37)	0.999	3 (4/141)
<i>Histophilus somni</i>	11 (7/67)	11 (4/37)	5 (2/37)	0.740	9 (13/141)
<i>Mannheimia</i> spp.	8 (5/67)	5 (2/37)	8 (3/37)	0.932	7 (10/141)
<i>Fusobacterium</i> spp.	49 (33/67) ^b	38 (14/37) ^b	8 (3/37) ^a	0.002	36 (50/141)
<i>Prevotella</i> spp.	45 (30/67) ^b	24 (9/37) ^a	22 (8/37) ^a	0.030	33 (47/141)
<i>Clostridium</i> spp.	36 (24/67)	16 (6/37)	22 (8/37)	0.082	27 (38/141)
<i>Bacteroides fragilis</i>	45 (30/67) ^b	22 (8/37) ^a	16 (6/37) ^a	0.007	31 (44/141)
<i>Porphyromonas</i> spp.	39 (26/67)	27 (10/37)	16 (6/37)	0.063	30 (42/141)

^{a,b}Values with different superscripts within a row denote significant differences ($P < 0.05$) between cows with 2, 1 or 0 signs of metritis.

¹Two signs = reddish-brown, fetid vaginal discharge and rectal temperature (RT) $\geq 39.5^{\circ}\text{C}$ within 21 DIM; 1 sign = reddish-brown, fetid vaginal discharge or RT $\geq 39.5^{\circ}\text{C}$ within 21 DIM; 0 signs = no discharge or non-fetid lochia and RT $< 39.5^{\circ}\text{C}$ within 21 DIM.

²*P*-value derived from binary logistic regression to evaluate the effect of signs indicative of acute puerperal metritis on frequencies of isolated pathogens.

DISCUSSION

Prudent use of antimicrobials in people and in live-stock animals is urgently required to address worldwide concerns about emerging resistances. Antibiotics that are used in severely ill humans especially, such as third-generation cephalosporins, should be used with great caution in food-producing animals, and the use of MIC to follow changes in the resistance pattern and possible emergence of resistance has been suggested (Oliveira et al., 2012). Therefore, the objective of this study was to assess MIC of commonly used antibiotics for the major APM-causing bacteria *E. coli* and *T. pyogenes* to support responsible and prudent use.

As a prerequisite, we investigated the frequency distribution of bacterial species associated with different clinical signs indicative of APM. Interestingly, *T. pyogenes* and *E. coli* were isolated from cows with or suspected to have APM and from cows without any signs of APM. This is in line with the finding that the development of uterine disease depends not only on the bacterial species present, but also on the immune response of the cow and the bacterial load (Sheldon et al., 2006). Moreover, a certain degree of inflammation is likely necessary to support physiological adaptations after parturition (Bradford et al., 2015). It was shown, however, that intrauterine infusion of *T. pyogenes* caused a massive inflammatory response, demonstrating the negative effect of this bacterium on uterine tissue (Lima et al., 2015). Our results also showed that *E. coli*, *T. pyogenes*, *Fusobacterium* spp., and *Bacteroides fragilis* were isolated more frequently from cows with 2 signs of APM (RT $\geq 39.5^{\circ}\text{C}$ and fetid, reddish-brown

vaginal discharge) compared with cows with no signs of APM. This is in accordance with observations that on d 10 after calving, the prevalence of *T. pyogenes*, *E. coli*, and *Bacteroides* spp. was greater in cows with fetid vaginal discharge than in healthy cows (Huszenicza et al., 1999). Furthermore, it has been shown that the presence of abnormal cervical discharge at d 1, 14, and 28 postpartum is positively associated with the presence of *T. pyogenes* (Dohmen et al., 2000). Also, the occurrence of *E. coli* and its endotoxins in lochia early postpartum promotes the development of uterine infections later postpartum (Dohmen et al., 2000). Moreover, Williams et al. (2005) found that the risk for fetid odor of vaginal mucus was increased when *T. pyogenes*, *E. coli*, non-hemolytic streptococci, and *Mannheimia haemolytica* were isolated. To sum up, our study supports the common finding that *E. coli* and *T. pyogenes* are likely to be found in cows with APM.

In the next step, we provided MIC data for *E. coli* and *T. pyogenes* isolated postpartum from indoor-housed cows of 7 commercial farms in Germany. In our study, the MIC₉₀ of ceftiofur for *T. pyogenes* was lower (0.5 $\mu\text{g/mL}$) than that reported by Santos et al. (2010a; 32 $\mu\text{g/mL}$) and de Boer et al. (2015; 2 $\mu\text{g/mL}$). Although the MIC₅₀ and MIC₉₀ from the latter study (de Boer et al., 2015) were only 2 dilutions greater than the MIC reported here, Santos et al. (2010a) described 3- and 7-fold-greater dilutions for MIC₅₀ and MIC₉₀. The small number of sampled cows combined with up to 8 isolates per sample used for MIC determination by Santos et al. (2010a), as well as differences in methodology, might have contributed to higher MIC in that study and, therefore, should be interpreted with

Table 2. Frequency distribution (% of all isolates; n = 37) of MIC for 16 antimicrobials against *Trueperella pyogenes* isolates from the uteri of cows with 2 signs indicative of metritis (n = 23), 1 sign indicative of metritis (n = 11), and without any sign of metritis (n = 3) on 6 (interquartile range: 5 to 7) DIM¹

Antimicrobial agent(s)	MIC (µg/mL)															MIC ₅₀ ²	MIC ₉₀ ²
	0.008	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	
Ciprofloxacin	0	0	0	0	0	0	2.7	45.9	51.4	0	0	0	0	0	0	0	2
Gentamicin	0	0	0	0	0	0	8.1	70.3	5.4	8.1	5.4	2.7	0	0	0	0	1
Enrofloxacin	0	0	0	0	0	0	13.5	83.8	2.7	0	0	0	0	0	0	0	4
Trimethoprim/sulfamethoxazole 1:19	5.4 ³	0	16.2	48.7	21.6	8.1	0	0	0	0	0	0	0	0	0	0	1
Marbofloxacin	0	0	0	0	0	0	5.4	29.7	64.9	0	0	0	0	0	0	0	0.12
Tetracycline	0	0	0	2.7 ³	0	0	16.2	32.4	2.7	0	2.7	0	27.1	10.8	0	0	2
Tilmicosin	0	0	75.7 ³	2.7	13.5	5.4	0	2.7	0	0	0	0	0	0	0	0	64
Tulathromycin	0	0	0	0	0	5.4	37.9	29.7	24.3	2.7	0	0	0	0	0	0	0.12
Ampicillin	0	81.1 ³	0	16.2	2.7	0	0	0	0	0	0	0	0	0	0	0	2
Amoxicillin/clavulanic acid 2:1	0	58.3 ³	0	38.9	2.8	0	0	0	0	0	0	0	0	0	0	0	0.06
Penicillin G	97.3 ³	0	2.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0.06
Ceftiofur	0	0	0	0	0	56.8	40.5	2.7	0	0	0	0	0	0	0	0	≤0.008
Ceftiofur	0	0	0	2.7	56.8	40.5	0	0	0	0	0	0	0	0	0	0	0.25
Cefquinome	0	0	70.3 ³	0	27.0	2.7	0	0	0	0	0	0	0	0	0	0	0.12
Cephalothin	0	0	16.2	40.6	32.4	10.8	0	0	0	0	0	0	0	0	0	0	0.25
Cefotaxim	0	0	54.1 ³	0	32.4	8.1	2.7	2.7	0	0	0	0	0	0	0	0	0.25
Cefoperazone	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.25

¹Tested concentrations of antimicrobials are framed by gray cells.

²Concentrations that inhibited growth of 50% and 90% of isolates, respectively.

³Isolates that did not grow at the lowest concentration of the antimicrobial tested.

Table 3. Frequency distribution (% of all isolates; n = 85) of MIC for 16 antimicrobials against *Escherichia coli* isolates from the uteri of cows with 2 signs indicative of metritis (n = 51), 1 sign indicative of metritis (n = 27), and without any sign of metritis (n = 7) on 5 (interquartile range: 3 to 7) DIM¹

Antimicrobial agent(s)	MIC (µg/mL)															MIC ₅₀ ²	MIC ₉₀ ²
	0.008	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	
Ciprofloxacin	2.4	61.3	29.1	1.2	1.2	2.4	0	0	0	0	0	2.4	0	0	0	0	0.03
Gentamicin	0	3.5	64.7	25.9	1.2	11.8	69.4	11.8	4.7	0	0	2.3	0	0	0	0	0.5
Enrofloxacin	0	0	9.4	41.2	27.0	11.8	1.2	0	0	0	0	0	2.3 ³	0	0	0	0.06
Trimethoprim/sulfamethoxazole 1:19	0	3.5	70.5	18.8	0	1.2	2.4	0	0	0	2.4	0	0	9.4 ³	0	0	0.06
Marbofloxacin	0	0	0	0	0	0	1.2	4.7	28.2	47.0	9.4	2.4	0	1.2	5.9	41.2 ³	0.06
Tetracycline	0	0	0	0	0	0	1.2	1.2	0	0	0	0	1.2	17.6	38.8	128	8
Tilmicosin	0	0	0	0	0	0	1.2	1.2	0	0	25.8	45.9	21.2	5.9 ³	0	0	≥256
Tulathromycin	0	0	0	0	0	0	1.2	0	0	0	5.9	0	0	0	0	0	32
Ampicillin	0	0	0	1.2	0	0	1.2	0	29.4	50.5	9.4	0	0	0	11.8 ³	0	≥128
Amoxicillin/clavulanic acid 2:1	0	0	0	1.2	0	0	2.4	14.1	69.3	9.4	1.2	9.4	2.4	0	0	0	8
Penicillin G	0	1.2	0	0	0	0	0	0	0	0	0	9.4	62.3	27.1 ³	5.9 ³	0	≥64
Ceftiofur	0	0	0	0	29.4	60.0	4.7	0	0	0	0	0	0	0	0	0	1
Cefquinome	0	15.2	64.7	7.0	2.4	1.2	0	2.4	2.4	1.2	0	0	0	0	0	0	0.5
Cephalothin	0	0	0	1.2	0	0	0	0	3.5	38.8	40.0	10.6	0	0	0	5.9 ³	0.06
Cefotaxim	1.2 ⁴	0	1.2	63.5	17.6	8.2	2.4	0	0	0	0	1.2	1.2	3.5 ³	0	0	0.25
Cefoperazone	0	1.2 ⁴	0	21.2	52.9	9.4	5.9	3.5	0	0	0	0	0	5.9 ³	0	0	1

¹Tested concentrations of antimicrobials are framed by gray cells.

²Concentrations that inhibited growth of 50% and 90% of isolates, respectively.

³Isolates that were not inhibited by the highest concentration of the antimicrobial tested.

⁴Isolates that did not grow at the lowest concentration of the antimicrobial tested.

Table 4. Frequency distribution (% of all isolates) of MIC of ceftiofur against *Trueperella pyogenes* and *Escherichia coli* isolates from the uteri of cows considering number of clinical signs indicative for acute puerperal metritis

Pathogen	No. of signs of metritis ¹	MIC (µg/mL)				MIC ₅₀ ²	MIC ₉₀ ²
		0.25	0.5	1.0	≥64		
<i>T. pyogenes</i>	2 (n = 23)	52.17	47.83	0	0	0.25	0.5
	1 (n = 11)	54.55	36.36	9.09	0	0.25	0.5
	0 (n = 3)	100.00	0	0	0	0.25	0.25
<i>E. coli</i>	2 (n = 51)	37.25	49.02	3.92	9.80	0.5	1.0
	1 (n = 27)	18.52	74.07	7.41	0	0.5	1.0
	0 (n = 7)	14.29	85.71	0	0	0.5	0.5

¹Two signs = reddish-brown, fetid vaginal discharge and rectal temperature (RT) ≥39.5°C within 21 DIM; 1 sign = reddish-brown, fetid vaginal discharge or RT ≥39.5°C within 21 DIM; 0 signs = no discharge or non-fetid lochia and RT <39.5°C within 21 DIM.

²Concentrations that inhibited growth of 50% and 90% of isolates, respectively.

caution. Analyzing MIC, however, was only a secondary outcome of Santos et al. (2010a). In contrast, the MIC₉₀ values for *T. pyogenes* reported in our study were greater than those from Sheldon et al. (2004; 0.125 µg/mL). In the absence of epidemiological or clinical interpretive criteria, interpretation of our MIC remains speculative. It has been reported, however, that mean concentrations of potentially active ceftiofur derivatives in serum, endometrium, and lochia after administration of the European Union-approved dosage of 1.1 mg/kg on 3 consecutive days remained greater than 0.5 µg/mL (or µg/mg) for the entire dosing interval (Drillich et al., 2006). This concentration exceeds the MIC₉₀ for *T. pyogenes* reported in our study. As with all β-lactams, the aim of treatment is to maintain serum and tissue concentrations of drug equal to or greater than the MIC for the majority of or the entire dosing interval (Prescott, 2013). Therefore, the label dose can be considered suf-

ficiently high to achieve therapeutic concentrations for *T. pyogenes* as analyzed in this study.

For *E. coli*, the MIC values reported by de Boer et al. (2015) and Sheldon et al. (2004) were lower (0.5 µg/mL) than our result (1 µg/mL); however, our value was comparable to that of Santos et al. (2010b; 1 µg/mL). The greater MIC for *E. coli* than *T. pyogenes* in our study is not surprising because gram-positive organisms (i.e., *T. pyogenes*) are more susceptible to β-lactams than gram-negative bacteria (i.e., *E. coli*), and MIC are lower for gram-positive bacteria (Papich, 2014). With a dosage of 1.1 mg/kg on 3 consecutive days, the mean maximum concentration of ceftiofur derivatives in serum and endometrium exceeded 1 µg/mL (or µg/mg) after each injection and in lochia after the second injection (Drillich et al., 2006). Twenty-four and 48 h after initial treatment, however, measured mean concentrations of ceftiofur were below 1 µg/mL in that study. The optimum duration of plasma concentrations above the MIC is considered to maintain at least 40 to 50% of the dosing interval (Turnidge, 1998; Toutain et al., 2002; Papich, 2014). As there was no continuous measurement of ceftiofur derivatives in blood or tissue in Drillich et al. (2006), it is not clear for how long the concentration exceeded the reported MIC.

Recently, ampicillin has been shown to achieve greater clinical cure rates than ceftiofur in cows with APM (Lima et al., 2014). Moreover, at a dose of 11 mg/kg of BW every 24 h for 3 consecutive days, ampicillin concentrated in lochial fluid compared with plasma and reached high median maximum concentrations after first injection of 55.7 µg/mL (Credille et al., 2015). For *T. pyogenes*, the MIC₉₀ of ampicillin determined in our study was low (0.06 µg/mL), with 81.1% of all isolates being inhibited at the lowest ampicillin concentration tested, which is in line with a previous report (de Boer et al., 2015; 0.06 µg/mL). In contrast, more than 30% of *T. pyogenes* isolates tested by Santos et al. (2010a)

Figure 1. Proportion of *Trueperella pyogenes* and *Escherichia coli* that are inhibited by increasing concentrations of ceftiofur.

Table 5. Summary of MIC of ceftiofur against *Trueperella pyogenes* and *Escherichia coli* from 5 studies

Pathogen	Study	No. of sampled cows	No. of analyzed isolates	MIC ₅₀ ¹ (µg/mL)	MIC ₉₀ ¹ (µg/mL)
<i>T. pyogenes</i>	Current study	141	37	0.25	0.5
	de Boer et al., 2015	575	35	1	2
	Santos et al., 2010a	10	72	2	32
	Sheldon et al., 2004	70	22	<0.06	0.125
<i>E. coli</i>	Current study	141	85	0.5	1
	de Boer et al., 2015	575	209	0.25	0.5
	Santos et al., 2010b	5	80	1	1
	Sheldon et al., 2004	70	20	0.5	0.5

¹Concentrations that inhibited growth of 50% and 90% of isolates, respectively.

were not inhibited at the highest ampicillin concentration tested, and the reported MIC₉₀ was ≥ 64 µg/mL. Regarding *E. coli*, 11.8% of tested isolates were not inhibited at the highest ampicillin concentration tested in the current study and the MIC₉₀ was ≥ 128 µg/mL, respectively. The MIC of ampicillin described by de Boer et al. (2015) was 4 µg/mL and that reported by Santos et al. (2010b) was 32 µg/mL. Reported median peak ampicillin concentrations at steady state were 5.27 µg/mL in lochial fluid, 3.11 µg/mL in plasma, and 1.55 µg/mL in endometrial tissue (Credille et al., 2015). This illustrates that concentrations of ampicillin in lochia, plasma, and endometrium exceed the MIC₉₀ of *T. pyogenes* analyzed in our study, whereas the MIC₉₀ of *E. coli* was not reached. In summary, *E. coli* with high MIC against ceftiofur as well as ampicillin were found in this study. Therefore, susceptibility testing before therapeutic decisions and prudent use of ceftiofur (to maintain it as the last choice for resistant cases of APM) is suggested. The use of other antimicrobials (e.g., ampicillin) should be considered first.

It is clear that many antimicrobials tested in this study showed a bimodal distribution of MIC values. Epidemiological cut-off values divide a wild-type population without acquired or mutational resistance mechanisms to a given drug and a non-wild-type population harboring such resistance mechanisms, and can be used to indicate development of microbiological resistance (Dalhoff et al., 2009). Visual inspection of MIC distributions showed that for *T. pyogenes*, tetracycline, tilmicosin, ampicillin, amoxicillin, cephalothin and cefoperazone had bimodal distributions, possibly indicating development of resistance against these agents. For *E. coli*, several tested antimicrobials (i.e., gentamicin, enrofloxacin, trimethoprim/sulfamethoxazole, marbofloxacin, tetracycline, ampicillin, and cephalosporins) also showed a bimodal distribution of MIC. Because *E. coli* is a gram-negative species, intrinsic resistance to some antimicrobials, rather than development of resistance, could explain the high MIC.

Finally, different clinical signs of APM have been assessed as variables potentially associated with MIC. We hypothesized that cows with APM might have pathogens with increased MIC. However, we did not find variation in the distribution of MIC values of ceftiofur for *T. pyogenes* and *E. coli* among the 3 study groups. As this is the first study to evaluate differences in MIC for bacteria isolated from healthy cows and those diagnosed with APM, comparable data are not available. Moreover, our results should be interpreted with caution because of the small number of isolates from cows without signs of APM ($n = 3$ and $n = 7$ for *T. pyogenes* and *E. coli*, respectively) because type II errors might have occurred. One study evaluated MIC of cows with either clinical or subclinical mastitis for *Staphylococcus aureus* (Oliveira et al., 2012). In that study, MIC of ceftiofur varied between clinical and subclinical cases, whereas differences in MIC for ampicillin were not found (Oliveira et al., 2012).

CONCLUSIONS

Few data on in vitro susceptibilities of uterus-borne bacteria are available. In this study, MIC of antimicrobials for *E. coli* and *T. pyogenes* isolated from cows from 7 farms in Germany were determined and compared with previous findings and with plasma and tissue distributions of ceftiofur and ampicillin. Minimum inhibitory concentrations of ceftiofur were low. However, not all *E. coli* could be inhibited at the highest concentration of ceftiofur tested, which might be an indicator of emerging resistance.

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