Minimum inhibitory concentrations of selected antimicrobials against *Escherichia coli* and *Trueperella pyogenes* of bovine uterine origin

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ABSTRACT

Minimum inhibitory concentrations (MIC) of 9 antimicrobials for isolates of 2 common bovine intrauterine bacterial pathogens, *Escherichia coli* (n = 209) and *Trueperella pyogenes* (n = 35), were determined using broth microdilution methodology. The isolates were recovered from dairy cows from 7 herds postpartum using the cytobrush technique. The pathogens were initially identified using phenotypic techniques. Additionally, PCR was used to confirm the identity of *T. pyogenes* isolates and to categorize the *E. coli* isolates into phylogenetic groups A, B1, B2, and D. Minimum inhibitory concentrations in excess of published cut-points or bimodal distributions of MIC indicated potential antimicrobial resistance to ampicillin, cefuroxime, cephapirin, and oxytetracycline for *E. coli*, and to oxytetracycline for *T. pyogenes*. Of the antimicrobials tested, ticarcillin/clavulanic acid, ceftiofur, and enrofloxacin had the lowest MIC for these 2 pathogens. Differences in MIC of some antimicrobials were found between herds, age, breeds, and *E. coli* phylogenetic groups. Isolation of *E. coli* with an MIC ≥ 8 $\mu\text{g/mL}$ of oxytetracycline at 23 d postpartum was associated with a lower probability of pregnancy within 6 wk of commencement of breeding compared with those isolates with an MIC < 8 $\mu\text{g/mL}$ (relative risk = 0.66). Minimum inhibitory concentrations for uterine pathogens were determined for isolates from New Zealand dairy cows. However, in the absence of either epidemiological or clinical interpretive criteria, the interpretation of these MIC remains unclear. Further studies are required to define interpretative criteria, including determination of pharmacokinetic and pharmacodynamic profiles for antimicrobials.

Key words: minimum inhibitory concentration, antimicrobial susceptibility, uterine infection, dairy cow

INTRODUCTION

A wide range of aerobic and anaerobic bacteria can be found in the postpartum uterus of cows (Santos et al., 2011). Many species have been isolated, including *Escherichia coli*, *Trueperella pyogenes*, *Prevotella* spp., *Fusobacterium necrophorum*, *Bacillus licheniformis*, *Clostridium perfringens*, *Pasteurella multocida*, *Staphylococcus* spp., and *Streptococcus* spp. (Williams et al., 2005). Commonly, multiple bacterial species are isolated from a single cow at a single time point. These bacteria have been classified into 3 different categories of pathogenicity, with *E. coli* and *T. pyogenes* purportedly pathogenic for the endometrium, whereas the others are classified as opportunistic or potential pathogens (Williams et al., 2005). In vitro and in vivo results suggest that isolation of *E. coli* after calving may predispose cows to infections with other bacteria or viruses (Dohmen et al., 2000; Donofrio et al., 2008).

Broad-spectrum antimicrobial therapy is usually recommended and deemed appropriate for uterine infections shortly after calving (Azawi, 2008), but its efficacy depends on pathogen susceptibility. A variety of methods are used to evaluate antimicrobial sensitivity of pathogens, including agar zone (or disc) diffusion assays, broth dilution methods or modifications thereof, and procedures to find penicillin-resistant genes (Watson et al., 1991; Salmon et al., 1995; Vintov et al., 2003). Compared with the zone diffusion system, the broth dilution method is less frequently used because it is more complex, time consuming, and expensive. Results of the zone diffusion and broth dilution methods are highly correlated (Watson et al., 1991). However, because zone diffusion assays only categorize laboratory outcomes as susceptible, intermediate susceptible, or resistant, it is not possible to quantify resistance by actual concentrations of the antimicrobial. The advantage of knowing the MIC (obtained by broth dilution) is that resistance can be quantified more accurately and, where required, antimicrobial concentrations can be modified to achieve therapeutic serum or tissue concentrations where phar-

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macokinetic or pharmacodynamic data are available (Turnidge and Paterson, 2007).

Interpretive cut-points for MIC are available from the Veterinary Antimicrobial Susceptibility Testing subcommittee of the Clinical and Laboratory Standards Institute (CLSI, 2013) for a range of veterinary pathogens and antimicrobials. Cut-points derived from human trials with human pathogens and antimicrobials, as determined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org), have also been used to assess veterinary antimicrobials (Bengtsson et al., 2009). However, no MIC interpretive criteria have been established for bovine uterine-derived *E. coli* or *T. pyogenes*.

Only a few studies have specifically examined the MIC of antimicrobials for pathogens isolated from the bovine uterus, particularly for *E. coli* (Sheldon et al., 2004; Santos et al., 2010b) and *T. pyogenes* (Sheldon et al., 2004; Liu et al., 2009; Santos et al., 2010a), and there are no data from isolates from pasture-based systems as commonly used in New Zealand. The primary aim of this study was to determine MIC for *E. coli* and *T. pyogenes* from pasture-based dairy cows and, second, to explore associations between MIC and demographic factors (such as herd and age) and reproductive performance.

MATERIALS AND METHODS

Herds, Cows, and Isolate Collection

Isolates were from cows from a convenience sample of 7 herds, in which cows calved on pasture in spring, were managed on pasture, and were predominantly fed on rye grass/white clover pasture. Breeding management included a period of 4 to 8 wk during which any cow detected in behavioral estrus was bred by AI, followed by a period of 6 to 9 wk where bulls were placed with the herd. Pregnancy diagnosis by transrectal ultrasonography occurred on 2 or 3 calendar days, and stage of gestation, and hence conception date, were determined. Cow data (e.g., calving date, age, breed, dates of AI) were recovered from industry electronic databases, and pregnancy diagnosis data were recorded onto hardcopy worksheets on the farm before being transcribed to a purpose-built database.

The isolates were collected as part of 2 studies. The isolates from study 1 were collected from 272 cows from 6 herds. That study had been established to test the hypotheses that isolation of *E. coli* in the early postpartum period increased the risk of infection with either *E. coli* or *T. pyogenes* approximately 3 wk later (de Boer et al., 2015). The cows were a random sample of cows

calving over a 1-wk period in each of the 6 herds. Cows were sampled twice at a mean ($\pm$ SD) of 2 ($\pm$ 1.2) and 23 ($\pm$ 1.2) DIM. Isolates from study 2 were obtained from cows ($n = 303$) from 1 herd sampled twice at a mean ($\pm$ SD) of 29 ($\pm$ 2.4) and 43 ($\pm$ 2.3) DIM (McDougall et al., 2011). That study was established to define the prevalence of endometritis based on a series of different case definitions. The isolates used in the current study included 1 isolate from each cow at each time point at which *E. coli* or *T. pyogenes* had been isolated.

The number of cows sampled in these 2 studies was defined based on the primary hypotheses being evaluated in the studies. A priori, the prevalence of *E. coli* and *T. pyogenes* and the MIC distributions were unknown for cows in New Zealand; hence, formal power and analysis had not been undertaken to define the sample size for the MIC evaluations or to evaluate the relationship between MIC and fertility outcomes.

Microbiology

The samples had been collected via a triple guarded cytobrush system (Barlund et al., 2008) by 1 of 2 operators within each study. Collection and storage of isolates have been previously described (McDougall et al., 2011; de Boer et al., 2015). In study 1, the cytobrushes were used to streak a 0.1% esculin–5% sheep blood agar plate and a MacConkey agar plate (Fort Richard, Auckland, New Zealand), and those collected in study 2 were used to streak 2 agar plates containing 0.1% esculin–5% sheep blood (Fort Richard). The MacConkey agar plate (study 1) and 1 of the esculin–sheep blood agar plates (study 2) were incubated for 2 d at 37°C in an aerobic environment, and the remaining esculin–sheep blood agar plates were incubated for 2 d at 37°C in sealed containers with CO₂. *Escherichia coli* and *T. pyogenes* isolates were selected based on colony morphology, shininess/glossiness, color, lactose fermentation (*E. coli*), hemolysis patterns, and Gram stain. Isolates were confirmed by a range of biochemistry tests: lactose, maltose, mannitol, motility, OUMI [an enzymatic test for *ortho*-nitrophenyl- β -galactosidase (ONPG), urase, motility, indol], Simmons citrate, and triple sugar iron tests for presumptive *E. coli* isolates, and lactose, maltose, mannitol, nutrient gelatin, and ONPG for presumptive *T. pyogenes* isolates. Each purified isolate was inoculated in 2 mL of brain-heart infusion broth with 20% glycerol and stored at –80°C for subsequent analysis. The identity of the *T. pyogenes* isolates from each study were confirmed by PCR using previously described primers (Jost et al., 2002; Hassan et al., 2008; Hijazin et al., 2011), whereas *E. coli* iso-

lates were divided into phylogenetic groups A, B1, B2, and D by triplex PCR (Clermont et al., 2000).

Susceptibility Testing

When more than one *E. coli* or *T. pyogenes* isolate was present for each sample time point, one isolate was randomly selected for susceptibility testing. In total, 209 *E. coli* and 35 *T. pyogenes* isolates were evaluated. The antimicrobial susceptibility tests were undertaken following guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013). A custom-made plate for the broth microdilution method (Sensititer, Trek Diagnostic Systems Ltd., East Grinstead, UK) was used for the susceptibility testing of ampicillin, cloxacillin, ticarcillin/clavulanic acid, ceftiofur, cefuroxime, cephalirin, enrofloxacin, clindamycin, and oxytetracycline. All antimicrobials were tested in a range from 0.0625 to 32 or 64 µg/mL in doubling dilutions (i.e., 10 or 11 dilutions). Before susceptibility testing, isolates from brain-heart infusion broth were revived on Mueller Hinton agar containing 5% sheep blood (Fort Richard) and incubated at 37°C, *E. coli* isolates aerobically for 24 h and *T. pyogenes* isolates in a CO₂-enriched environment for 48 h. Revived colonies were emulsified in 5 mL of demineralized water (Trek Diagnostic Systems Ltd.) to a turbidity of 0.5 McFarland standard. In total, 15 µL of the suspension was mixed in 11 mL of Mueller Hinton broth (Trek Diagnostic Systems Ltd.). Each well of the plate was inoculated with 50 µL of inoculated Mueller Hinton broth. For the *T. pyogenes* isolates, before this step, 2% lysed horse blood was added to the Mueller Hinton broth to support growth of these fastidious isolates (CLSI, 2013). Plates were incubated at 37°C for 24 and 48 h for *E. coli* and *T. pyogenes* inocula, respectively. Incubation times specified by the CLSI guidelines were increased to 48 h for *T. pyogenes* inocula due to the fastidious growth of these bacteria as previously recommended (Sheldon et al., 2004). The

well containing the lowest concentration without visible growth was classified as the MIC for that isolate.

To assess the microbial counts (cfu/mL) of the inocula, 1 µL of undiluted and 1 µL of 1:50 diluted inocula of the reference cultures and some unknown inocula were streaked over entire Mueller Hinton agar plates and incubated at 37°C for 24 and 72 h for *E. coli* and *T. pyogenes*, respectively, and the number of colonies was counted.

Before analysis of any wild-type isolates, MIC were determined for 20 replicates of the reference *E. coli* (ATCC 25922) and 40 replicates of the reference *T. pyogenes* (ATCC 19411). Additionally, a reference culture was tested in parallel with each group of wild-type isolates tested. Available reference ranges for the reference type cultures provided by the microdilution plate manufactures (Trek Diagnostic Systems Ltd.) were used as the normal ranges. For the antimicrobials (i.e., cloxacillin and clindamycin for the reference strain *E. coli* ATCC 25922 and all antimicrobials for the *T. pyogenes* ATCC 19411 reference strain) where no reference range data from either the broth microdilution plate manufacture or CLSI were available, reference ranges were calculated as previously described (Turnidge and Bordash, 2007; Table 1).

Statistical Analysis

The concentrations that inhibited 50% (MIC₅₀) and 90% (MIC₉₀) of the isolates were calculated for each antimicrobial. Minimum inhibitory concentrations were graphed and tabulated. Where interpretive cut-points for MIC have been established for some pathogens and antimicrobials by CLSI and EUCAST, these were used to interpret the results of the current study. Where no interpretative cut-points were available from CLSI or EUCAST, the MIC were plotted on frequency histograms and normality of distribution was tested using Shapiro-Wilk test (Razali and Wah, 2011). When dis-

Table 1. The Clinical and Laboratory Standards Institute (CLSI) and broth microdilution plate (Sensititer, Trek Diagnostic Systems Ltd., East Grinstead, UK) suggested and calculated diagnostic reference ranges (µg/mL) for *Escherichia coli* and *Trueperella pyogenes* ATCC type cultures used as quality control

Antimicrobial	<i>E. coli</i> ATCC 25922			<i>T. pyogenes</i> ATCC 19411
	CLSI	Trek	Calculated	Calculated
Ampicillin	2–8	2–8	—	0.03–0.125
Cloxacillin	—	—	32–128	0.25–1
Ticarcillin/clavulanic acid	4/2–16/2	4/2–16/2	—	0.03–0.125
Ceftiofur	0.25–1	0.25–1	—	0.5–2
Cefuroxime	—	2–8	—	0.03–0.125
Cephapirin	—	8–32	—	0.06–0.25
Enrofloxacin	0.008–0.03	0.008–0.03	—	0.5–2
Clindamycin	—	—	32–128	0.03–0.125
Oxytetracycline	—	0.5–2	—	0.125–0.5

tribution was not normal and clearly a bimodal distribution, cut-points were visually created.

Further analyses were undertaken on a subset of *E. coli* (n = 175) and *T. pyogenes* (n = 26) isolates from study 1. Associations between MIC values (log₂-transformed) with herd, age (categorized as 3–4, 5–6, and ≥ 7 yr), breed (categorized as Friesian, Jersey, and cross-breed), sample day (2 or 23 DIM), and phylogenetic grouping for *E. coli* were assessed using Fisher’s exact analyses. Binary logistic regression was undertaken to evaluate the association between MIC of antimicrobials and reproductive performance. The outcome variables were submission for AI within 3 wk after the start of the seasonal breeding program (planned start of mating; **PSM**), pregnant within 3 or 6 wk after PSM, and conception to first AI within 3 wk after PSM. The MIC of each antimicrobial separately (categorized by binary cut-point available from CLSI, EUCAST; Table 2, or from a possible bimodal distribution) was included in each model to evaluate the causal effect on each outcome variable. Herd was included as a fixed effect in each model. Variables were retained if the *P*-value was ≤ 0.10, or if it caused a change in one of the coefficients by >20%. Relative risk was calculated using Poisson regression. Data analysis was undertaken using Stata 13.1 (StataCorp, College Station, TX).

RESULTS

Quality Assurance

A total of 27 *E. coli* ATCC 25922 and 43 *T. pyogenes* ATCC 19411 reference strains were tested, and of the 189 individual tests, 2 (1.1%) were not within the available reference ranges. For those antimicrobials for which no reference range data were available, 8 (1.8%) of the 441 individual tests were not within the calculated reference ranges (Table 1). Determinations of MIC were repeated for 12 of 244 (5%) wild-type isolates as growth in the plate was not acceptable (i.e., >1 “skips” of growth within a sequence of dilutions within an antimicrobial occurred) or the positive controls had no growth. A total of 92.3% (n = 36/39) of inocula were between 5 × 10⁴ and 5 × 10⁵ cfu/mL, the optimum inoculum (CLSI, 2013).

Frequency Distribution and MIC₅₀ and MIC₉₀

Ampicillin and oxytetracycline had the highest MIC and the widest range (1 to ≥128 μg/mL) for *E. coli*, whereas enrofloxacin had the lowest MIC detected (≤0.25 μg/mL). The MIC were ≥64 μg/mL for cloxacillin and clindamycin for *E. coli* as no isolates were inhibited, even at the highest concentration tested. The

Table 2. Frequency distribution (% of all isolates; n = 209) of MIC for a range of antimicrobials against *Escherichia coli* isolated between 2 (±1.2; SD) and 43 (±2.3) DIM from postpartum bovine uteruses¹

Antimicrobial	MIC (μg/mL)															NS% ²	MIC ₉₀	MIC ₅₀
	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128						
Ampicillin	0	0	0	0	2.9	23.4	69.4	1.9	0.5	0.5	0	1.4	4	4	1.9			
Cloxacillin	0	0	0	0	0	0	0	0	0	0	100	0	64	64	0			
Ticarcillin/clavulanic acid	0	0	0	0	9.1	67.5	22.0	0	1.4	0	0	0	2	4	0			
Ceftiofur	0	1.0	52.2	44.5	1.9	0.5	0	0	0	0	0	0	0.25	0.5	0			
Cefuroxime	0	0	0	0	0.5	11.5	76.1	10.5	0.5	0	1.0	0	4	8	1.4			
Cephapirin	0	0	0	0	0	1.4	7.7	26.8	57.4	6.2	0.5	0	16	16	6.7			
Enrofloxacin	97.1	1.9	1.0	0	0	0	0	0	0	0	0	0	0.06	0.06	0			
Clindamycin	0	0	0	0	0	0	0	0	0	0	100	0	64	64	0			
Oxytetracycline	0	0	0	0	6.2	41.1	35.9	12.0	0	0.5	0.5	3.8	4	8	4.8			

¹The light gray shading represents the intermediate susceptible zone, and the darker gray shading represents the resistant zone as suggested by the Clinical and Laboratory Standards Institute or European Committee on Antimicrobial Susceptibility Testing. Antimicrobials without shading have no interpretive criteria available. Those isolates not inhibited at the highest concentration of antimicrobial tested are presented in the next highest dilution and reported as equal to or greater than that MIC. Where there are results in the lowest concentration tested, the results are reported as less than or equal to that MIC; MIC₅₀ and MIC₉₀ = MIC (μg/mL) that inhibited 50 and 90% of the isolates, respectively.

²NS% = percentage of nonsusceptible *E. coli* for antimicrobials using the interpretative criteria for *E. coli* isolated from other host species (i.e., humans and poultry) or from bovine isolates from other infection sites (i.e., mastitis).

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frequency distributions of MIC for *E. coli*, including the MIC₅₀ and MIC₉₀, are presented in Table 2. In total, 1.9, 1.4, 6.7, and 4.8% of the *E. coli* isolates were resistant (based on the interpretive criteria from human-derived data) for ampicillin, cefuroxime, cephalirin, and oxytetracycline, respectively (Table 2).

All antimicrobials inhibited growth of *T. pyogenes* isolates by at least one of the concentrations tested, except for oxytetracycline where 2.9% of the isolates were not inhibited at the highest concentration tested. Oxytetracycline also had the widest range (0.25 to ≥ 128 $\mu\text{g/mL}$) for *T. pyogenes*, whereas ampicillin and ticarcillin-clavulanic acid had the lowest MIC detected (≤ 0.13 $\mu\text{g/mL}$). The frequency distribution of MIC for *T. pyogenes*, including the MIC₅₀ and MIC₉₀, are presented in Table 3. In contrast to *E. coli*, no CLSI or EUCAST interpretive criteria are available for *T. pyogenes* isolated from any host species (including humans) or infection site.

Variables Associated with MIC

Variation in distribution of MIC values of some antimicrobials for *E. coli* and *T. pyogenes* among herds, age groups, and breeds were found (Table 4). The heterogeneity in MIC of ceftiofur ($P = 0.01$) and oxytetracycline ($P = 0.05$) observed among herds are presented in frequency distribution diagrams (Figure 1a and b, respectively). Differences ($P = 0.04$) among age groups and MIC values of ceftiofur for *T. pyogenes* are shown in Figure 2. Additionally, differences ($P \leq 0.05$) were found between the distribution of MIC of some antimicrobials for *E. coli* and sample day and genetic differentiation into phylogenetic groups (Table 4; Figure 3).

Of all MIC cut-points tested, only *E. coli* isolates at 23 DIM with an MIC ≥ 8 $\mu\text{g/mL}$ of oxytetracycline (6/10; 60%) tended to be less likely (relative risk = 0.66; 95% CI: 0.38–1.15; $P = 0.09$) to conceive within 6 wk than those with MIC < 8 $\mu\text{g/mL}$ (39/50; 78%).

DISCUSSION

This study has provided MIC data of 9 antimicrobials for 2 common bovine intrauterine pathogens, *E. coli* and *T. pyogenes* (Williams et al., 2005). Limited data are available on MIC values for these pathogens from the postpartum bovine uterus, and this is the first time these data have been generated for isolates of intrauterine origin from pasture-based cattle. Those cows may be exposed to different bacterial species and strains because they calved on pasture rather than in a housed environment. For example, pasture-based cows have a lower prevalence of mastitis caused by *E. coli* compared

Table 3. Frequency distribution (% of all isolates; n = 35) of MIC for a range of antimicrobials against *Trueperella pyogenes* isolated between 2 (± 1.2 ; SD) and 43 (± 2.3) DIM from postpartum bovine uterus¹

Antimicrobial	MIC ($\mu\text{g/mL}$)														MIC ₅₀	MIC ₉₀
	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128				
Ampicillin	97.1	2.9	0	0	0	0	0	0	0	0	0	0	0.06	0.06		
Cloxacillin	14.3	5.7	48.6	22.9	5.7	0	2.9	0	0	0	0	0	0.25	0.5		
Ticarcillin/clavulanic acid	91.4	8.6	0	0	0	0	0	0	0	0	0	0	0.06	0.06		
Ceftiofur	0	0	11.4	14.3	48.6	22.9	2.9	0	0	0	0	0	1	2		
Cefuroxime	74.3	17.1	5.7	2.9	0	0	0	0	0	0	0	0	0.06	0.12		
Cephapirin	5.7	28.6	62.9	2.9	0	0	0	0	0	0	0	0	0.25	0.25		
Enrofloxacin	0	0	0	34.3	65.7	0	0	0	0	0	0	0	1	1		
Clindamycin	85.7	8.6	5.7	0	0	0	0	0	0	0	0	0	0.06	0.12		
Oxytetracycline	0	0	20.0	48.6	22.9	2.9	0	0	2.9	0	0	2.9	0.5	1		

¹Those isolates not inhibited at the highest concentration of antimicrobial tested are presented in the next highest dilution and reported equal to or greater than that MIC. Where there are results in the lowest concentration tested, the results are reported as less than or equal to that MIC. There are no interpretive criteria available from the Clinical and Laboratory Standards Institute or European Committee on Antimicrobial Susceptibility Testing for antimicrobials against *T. pyogenes*; MIC₅₀ and MIC₉₀ = MIC ($\mu\text{g/mL}$) that inhibited 50 and 90% of the isolates, respectively.

Table 4. Probability values of Fisher's exact analyses for the bivariate associations between herd (n = 6), age (3–4, 5–6, and ≥7 yr), breed (Friesian, Jersey, and crossbreed), DIM at sampling [2 ± 1.2 (mean $\pm$ SD) and 23 ± 1.2 DIM], and genetic differentiation of pathogen into phylogenetic group (phylo group; A, B1, B2, and D) with the distribution of MIC of a range of antimicrobials for *Escherichia coli* (n = 175) and *Trueperella pyogenes* (n = 26) of bovine intrauterine origin

Pathogen and antimicrobial	Herd	Age	Breed	DIM	Phylo group
<i>E. coli</i>					
Ampicillin	0.38	0.65	0.18	0.30	<0.01*
Cloxacillin	— ¹	—	—	—	—
Ticarcillin/clavulanic acid	0.39	0.46	0.68	0.84	0.03*
Ceftiofur	0.01*	0.72	0.82	0.91	<0.01*
Cefuroxime	0.18	0.51	0.87	0.42	0.02*
Cephapirin	0.18	0.05*	0.38	0.05*	<0.01*
Enrofloxacin	1.00	1.00	1.00	0.35	1.00
Clindamycin	—	—	—	—	—
Oxytetracycline	0.05*	0.16	0.09†	0.60	0.03*
<i>T. pyogenes</i>					
Ampicillin	0.60	1.00	0.56	0.88	NA ²
Cloxacillin	0.83	0.65	0.19	0.32	NA
Ticarcillin/clavulanic acid	0.01*	0.30	0.01*	0.67	NA
Ceftiofur	0.93	0.04*	0.65	0.81	NA
Cefuroxime	0.08†	0.84	0.84	0.65	NA
Cephapirin	0.98	0.75	0.75	0.12	NA
Enrofloxacin	0.48	0.59	0.77	0.24	NA
Clindamycin	0.62	0.82	0.23	1.00	NA
Oxytetracycline	0.16	0.10†	0.66	0.72	NA

¹— = all isolates have the highest MIC tested (≥ 64 $\mu\text{g/mL}$).

²NA = not available, because no genetic differentiation was done for *T. pyogenes*.

† $P \leq 0.10$; * $P \leq 0.05$.

with those in housed systems (McDougall, 2002). Additionally, associations between the MIC of intrauterine pathogens and cow demographics (such as herd and age) and between MIC and reproductive outcomes have not been published before for any management system.

The MIC₅₀ and MIC₉₀ values reported in this study differ from those from previous studies in some instances. For ceftiofur, the MIC for *E. coli* isolates were similar to those of previous studies (Sheldon et al., 2004; Santos et al., 2010b). However, for ampicillin and oxytetracycline, the MIC found in this study were 2 to 3 dilutions lower than those reported by others (Sheldon et al., 2004; Santos et al., 2010b), and for cephapirin, the MIC₅₀ was 2 dilutions higher and MIC₉₀ was 1 dilution higher compared with MIC reported by Sheldon et al. (2004). The MIC of ceftiofur and enrofloxacin for *T. pyogenes* described in the current study were similar to concentrations reported in the United Kingdom, United States, and China, whereas MIC for ampicillin, clindamycin, and oxytetracycline were lower in the current study than in those studies (Sheldon et al., 2004; Liu et al., 2009; Santos et al., 2010a). In contrast, the MIC for cephapirin were 2 dilutions higher compared with MIC previously reported from *T. pyogenes* of uterine origin (Sheldon et al., 2004). The differences between MIC₅₀ and MIC₉₀ of bacteria from pasture-based cows and cows calving in pens may be attributable to laboratory or cow factors.

It is important to realize that regardless of management system, in vivo antimicrobial concentrations (i.e., pharmacokinetics) need to be above the MIC to have therapeutic effects, and therefore these differences may be clinically less meaningful.

Veterinary interpretation criteria of MIC are lacking for many antimicrobials. For example, only ceftiofur has veterinary-specific interpretative criteria for *E. coli* isolates from bovine origin; however, these criteria have not been validated for uterine isolates. Enrofloxacin has veterinary-specific interpretative criteria for *E. coli* isolates of poultry origin, and cut-points for other antimicrobials (Table 2) were derived from human data (CLSI, 2013). Even where veterinary cut-points from animal trials were available, it is likely that human data (e.g., human pharmacokinetics) have been used to calculate cut-points for veterinary use (Turnidge and Paterson, 2007). Therefore, in the absence of pharmacokinetics and pharmacodynamics data and clear evidence of a bimodal distribution of the MIC of these antimicrobials, no inferences can be drawn about the resistance in these isolates to the antimicrobials tested.

However, if potential resistance of *E. coli* isolates against the first-generation cephalosporin cephapirin were confirmed, this would be of concern. Cephapirin is the commonly used first-line antimicrobial for uterine infections in New Zealand, and our data indicate that at least some *E. coli* isolates may have elevated MIC for

Figure 1. Distribution (percentage of isolates within herd) of the MIC of (a) ceftiofur and (b) oxytetracycline for *Escherichia coli* of intra-uterine origin (n = 175) by herd.

Figure 2. Distribution (percentage of isolates within age category) of the MIC of ceftiofur for *Trueperella pyogenes* of intrauterine origin (n = 26) categorized by age.

this antimicrobial. As *E. coli* is present >3 wk postpartum in a proportion of New Zealand cows (McDougall et al., 2011; de Boer et al., 2015) and as some of these cows may be diagnosed with purulent vaginal discharge postpartum, at least some cows with an elevated MIC are likely to be treated with cephalosporins. In the current study, no associations were found between an elevated MIC for cephalosporins and any of the reproductive outcomes tested, suggesting that the MIC observed were not of biological significance. No pharmacokinetic data are available for cows following intrauterine infusion of cephalosporins. However, simple calculations (i.e., mass of cephalosporin infused/uterine luminal volume) suggest that current on-label cephalosporin treatment will likely achieve concentrations above the MIC reported in the current study. Additionally, it is not clear what effect uterine inflammation would have on the concentrations of antimicrobials following intrauterine infusion. Studies have shown that concentrations of some antimicrobials may be higher in infected tissue than in noninfected tissue (Clarke et al., 1996). Further studies are required to determine if the approximately 20% of cows found to have purulent vaginal discharge 14 d posttreatment with cephalosporins (McDougall et al., 2013) are associated with *E. coli* with elevated MIC for cephalosporins, or are related to presence of other bacterial species, due

to reinfection, or due to ongoing inflammation in the absence of bacteria.

No interpretative criteria are available for any of the evaluated antimicrobials against *T. pyogenes*, and clinicians are thus unable to determine optimal doses and frequencies for these compounds when treating uterine and other infections. None of the antimicrobials tested had an MIC₉₀ of <1 µg/mL against both *E. coli* and *T. pyogenes*. Ceftiofur is a third-generation cephalosporin, and relatively low MIC against both pathogens were found in the current study. It has been used in several studies assessing efficacy for uterine infections, particularly for early postpartum metritis (Drillich et al., 2001; Risco and Hernandez, 2003) and cows with grossly evident purulent discharge in the vagina (Kaufmann et al., 2010). In all these studies, ceftiofur was administered by subcutaneous injection. None of the *E. coli* isolates and 2.9% of *T. pyogenes* isolates had MIC greater than the reported maximum mean concentration of ceftiofur (≤2 µg/g) in endometrial tissue in early postpartum cows (Okker et al., 2002; Drillich et al., 2006). However, in cows with uterine disease, the highest mean concentration (2.02 µg/g) occurred 50 h after the initial start of treatment; that is, 2 h after the third administration of ceftiofur hydrochloride (1 mg/kg; Drillich et al., 2006). The compliance in completing courses of antimicrobial

Figure 3. Distribution (percentage of isolates) of the MIC for several antimicrobials for *Escherichia coli* stratified by phylogenetic group.

therapy is reportedly low, with only 24% of surveyed dairy producers in the United States completing antimicrobial treatment courses (Sawant et al., 2005). The mean concentrations of ceftiofur in endometrial tissue of cows with uterine disease and treated once daily for only 2 d was $<1.5 \mu\text{g/g}$ (Drillich et al., 2006). Hence, had cows been treated for only 2 d, 0.5% of *E. coli* isolates and 25.8% of *T. pyogenes* isolates in the current study would be expected to have an MIC above the maximum concentration achieved with this treatment regimen. Additionally, the highest mean concentration of ceftiofur of $2.33 \pm 1.63 \mu\text{g/g}$ was reached in endometrial tissue of healthy cows treated with the long-acting antimicrobial drug ceftiofur crystalline free acid (6.6 mg/kg s.c.) at 24 h after initial treatment, whereas 3 to 7 d posttreatment, all mean concentrations were $<2 \mu\text{g/g}$ (Witte et al., 2011). To our knowledge, no data on the pharmacokinetics or clinical efficacy following intrauterine infusion of ceftiofur are publicly available. Additionally, combinations of systemic and intrauterine infusion treatment may result in a better distribution of antimicrobials across all layers of the uterus (Braun et al., 2009). Hence, substantially more research is required on the pharmacokinetics, pharmacodynamics, and clinical efficacy of ceftiofur used in this way. At present, this would be an off-label use for the currently commercially licensed products.

Ticarcillin is a carboxypenicillin susceptible to β -lactamases; hence, it is used in combination with clavulanic acid. It has been used for therapy of endometritis in humans (Faro, 1991) and horses (LeBlanc, 2009). Intrauterine infusion of 1 or 3 g of ticarcillin results in relatively low ($<4 \mu\text{g/mL}$) serum concentrations, indicating little systemic absorption in the mare (Threlfall and Keefe, 1983). Intrauterine infusion, compared with i.v. administration, resulted in higher endometrial tissue concentration in the mare; however, the half-life of the formulation used was relatively short (Van Camp et al., 2000). There appear to be no studies on the clinical efficacy of ticarcillin for uterine infections in cattle.

The MIC values of some antimicrobials differed between herds, age groups, and breeds. This has been described before for bacteria isolated from milk samples (McDougall et al., 2014), but not for those of uterine origin. Differences in clinical severity, the diagnostic approach, antimicrobial therapeutic choices, or culling decisions may have accounted for the observed differences in MIC among herds and age groups. The reason for the difference between breeds and the MIC values of ticarcillin/clavulanic acid for *T. pyogenes* is unclear. Differences in *T. pyogenes* virulence factors have been found between isolates from different cows (Santos et al., 2010a). Variation in immune response among cow breeds might result in selection of *T. pyogenes* with

varying virulence and microbial resistance factors. In the current study, *E. coli* was differentiated into phylogenetic groups, and these were associated with MIC values for many of the tested antimicrobials. However, we found no clear pattern among the phylogenetic groups in terms of MIC between the various antimicrobials. Additionally, we found no association between phylogenetic group and reproductive performance (de Boer et al., 2015) so the biological significance of the variation in MIC among phylogenetic groups remains to be determined. In contrast, one study found no difference between *E. coli* phylogenetic groups and disk diffusion results (Sheldon et al., 2010). The heterogeneity in MIC observed among herds suggests that sensitivity testing of uterine isolates may need to be considered for individual herds before prescribing decisions are made.

The only elevated MIC with a potential association with impaired reproductive performance was $\geq 8 \mu\text{g/mL}$ of oxytetracycline for *E. coli* isolates. This cut-point was derived from human isolates for intermediate susceptible ($8 \mu\text{g/mL}$) and resistant ($\geq 16 \mu\text{g/mL}$) *E. coli* isolates (CLSI, 2013). Reproductive tract infections in early lactation are commonly treated with oxytetracycline (Azawi, 2008). Intrauterine treatment of oxytetracycline by infusion or solid form (i.e., pessaries) appears to result in a poor distribution to serum and milk (Bretzlaff et al., 1983; Roncada et al., 2000), suggesting that the therapeutic compound is retained within the lumen and endometrial tissue (Azawi, 2008). The mean concentrations of oxytetracycline in the endometrium as described in two 30-yr-old studies were approximately $23 \mu\text{g/g}$ after intrauterine infusion (Bretzlaff et al., 1983) and $\leq 4 \mu\text{g/g}$ after intramuscular injection (Maseri et al., 1980). Nonetheless, in the current study, a cut-point of $\geq 8 \mu\text{g/mL}$ of oxytetracycline may be clinically significant, suggesting that oxytetracycline may not be the drug of choice in reproductive tract infections, with 16.8% of the *E. coli* isolates above this criterion. The current study had limited power to detect the effect of resistant isolates on reproductive performance. If it is assumed that 65% of cows from which nonresistant *E. coli* or *T. pyogenes* were isolated became pregnant in the first 6 wk of the seasonal breeding program, that 10% of the isolates were resistant, and that the presence of a resistant pathogen resulted in an absolute 20% decline in the proportion of cows pregnant in 6 wk (i.e., 65% vs. 45% of cows pregnant in the first 6 wk of the seasonal breeding program in cows that had sensitive vs. resistant isolates), then a total of about 450 culture-positive cows would be required to find a significant difference ($P < 0.05$) with a power of 0.8. In the current study, *E. coli* was isolated from 175 cows; hence, the power to detect effects of resistant pathogens on reproductive performance was

<0.35. Thus, the inference drawn is not that there was no association between the MIC of intrauterine isolates and subsequent fertility, but rather that the sample size was insufficient to reach a firm conclusion about such associations, other than for the association between elevated MIC for oxytetracycline for *E. coli* and pregnancy rate. At the time these studies were designed, the prevalence of intrauterine infection with *E. coli* and *T. pyogenes*, the MIC distributions, and the prevalence of resistance of these bacterial species were unknown for pasture-based cows. Hence, formal power analyses were not undertaken. This study is the first to evaluate these outcomes and, although the sample size is limited, it provides data from which larger studies could be designed. Larger studies are required to determine the effects of resistance of intrauterine pathogens on reproductive performance.

CONCLUSIONS

Limited data are available on MIC of antimicrobials against pathogens of bovine uterine origin. This study provided MIC values of a range of antimicrobials for *E. coli* and *T. pyogenes* of intrauterine origin. This is the first study to report these data from pasture-based cows, and some differences were found compared with studies using cows in housed systems. The absence of interpretive criteria for these bovine isolates and of pharmacokinetic and pharmacodynamic data limits interpretation. Thus, further work is required on susceptibility testing of antimicrobials for use in veterinary medicine, in particular for bovine reproductive tract infections. In addition, future study designs should evaluate cows from multiple herds and age groups, because differences in MIC were found among herds and age groups. Finally, only *E. coli* isolates with a high MIC (≥ 8 µg/mL) for oxytetracycline had poorer reproductive performance compared with cows from which *E. coli* with lower MIC were isolated. Validation of MIC cut-points using reproductive outcomes may be more clinically meaningful in bovine reproductive tract infections compared with cut-points selected using human pharmacokinetic or pharmacodynamic data.

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