Relationship between *in vitro* susceptibility of bovine subclinical mastitis isolates and bacteriological outcome of intramammary treatment with cefquinome

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Abstract

The objective of the present study was to determine whether there was an association between the *in vitro* antimicrobial susceptibility test results of subclinical mastitis pathogens and bacteriological outcomes of intramammary treatments using cefquinome. A total of 110 intramammary pathogens from 51 cows were assessed in this study. Most intramammary infections were due to coagulase-negative staphylococci, environmental streptococci, and coliforms. The antimicrobial susceptibility to cefquinome was determined using the Kirby-Bauer disc diffusion method. Bacteriological cure rates for the sensitive, intermediate, and resistant isolates in the standard treatment group (three intramammary infusions of 75 mg cefquinome at 16 h intervals) were 82.4%, 90%, and 87.5%, respectively. These figures in the extended treatment group (six intramammary infusions of 75 mg cefquinome at 16 h intervals) were 83.3%, 100%, and 100%, respectively. Treatment outcomes were not associated with the results of sensitivity tests in the standard group. However, in the extended group, the probability of a bacteriological cure was lower in quarters from which cefquinome-sensitive pathogens were isolated than the quarters from which intermediate or resistant pathogens were isolated. Based on this study, the Kirby-Bauer susceptibility test result is a poor predictor for the bacteriological cure of subclinical mastitis treated with intramammary cefquinome.

Introduction

Antibacterial therapy is an important part of every mastitis control program in dairy cattle (Erskine *et al*., 2003). *In vitro* antimicrobial susceptibility tests of clinical or subclinical mastitis pathogens are frequently used by bovine practitioners to guide treatment decisions at the level of both the cow and herd. However, for certain antimicrobial agents, previous studies failed to demonstrate statistically significant correlations between the results of susceptibility testing and treatment outcomes for clinical and/or subclinical mastitis (Owens *et al*., 1997; Cattell *et al*., 2001; Constable and Morin, 2002; Hoe and Ruegg, 2005; Apparao *et al*., 2009 a & b). Cefquinome is a fourth-generation cephalosporin which has been developed solely for veterinary use. It has shown excellent *in vitro* and *in vivo* activity against a variety of animal pathogenic Gram-positive and Gram-negative bacteria (Limbert *et al*., 1991; Chin *et al*., 1992; Murphy *et al*., 1994; Bottner *et al*., 1995). The objective of the present study was to determine whether there was an association between the *in vitro* antimicrobial susceptibility test results of subclinical mastitis bacterial pathogens and bacteriological cure rates of intramammary (IMM) treatment with the use of cefquinome.

Materials and Methods

The study was conducted in the summer of 2007 in a closed, commercial, large Holstein dairy farm in the Tehran province of Iran, with an average of 1,100 lactating dairy cows. All lactating cows were milked thrice daily. The herd had a relatively low prevalence of *Staphylococcus aureus* (*S. aureus*) and was free of *Streptococcus agalactiae* and *Mycoplasma bovis* intramammary infections based on several individual and bulk tank milk cultures and serological tests.

Mastitis pathogens were obtained from a randomized controlled clinical trial that evaluated the efficacy of standard and extended cefquinome intramammary therapy for the treatment of persistent

subclinical mastitis (lasting at least 1 mo) in lactating dairy cows at various stages of lactation (Kasravi *et al*., submitted). Fifty-one dairy cows with 110 infected quarters were enrolled in the study based on composite milk Somatic Cell Count (SCC) ⩾150,000 /ml at the last test-day record, positive California Mastitis Test (CMT) scores (scores T, 1, 2, and 3) at the time of first pre-treatment sampling, quarter milk SCC ⩾200,000 /mL and isolation of the same mastitis pathogen in the two samples obtained 7 d apart. CMT was used as a cow side screening test to prevent quarters with low scores from entering the experiment before knowing both the SCC and culture results. Sampling and microbiological procedures were conducted in accordance with standards of the National Mastitis Council (NMC) (Oliver *et al*., 2004).

Infected cows (not quarters) were grouped by parity and days in milk and randomly allocated into treatment groups. The infected quarters of cows enrolled in the study were treated by IMM infusion of 75 mg of cefquinome (Cefquinome sulphate; Cobactan LC, Intervet International, Holland) three times at 16 h intervals according to the recommendations of the manufacturer for herds that are milked three times a day (standard regimen group: 25 infected cows, 52 Intra Mammary Infection (IMI)), or six times at 16 h intervals (extended regimen group: 26 infected cows, 58 IMI). A negative untreated control group was also included (22 cows, 40 IMI).

Mueller-Hinton medium (Merck, Darmstadt, Germany) was used for the sensitivity testing of Gram-negative bacteria and *Staphylococci* spp., and Mueller-Hinton medium supplemented with 5% defibrinated sheep blood was used for sensitivity testing of Streptococci and *Corynebacteria* spp. The mastitis isolates were evaluated for antimicrobial sensitivity via the Kirby-Bauer disc diffusion method. The procedure was performed in accordance to CLSI guidelines (Clinical Laboratory Standards Institute, 2007). Disks impregnated with 10 µg cefquinome sulphate were used to determine the susceptibility pattern (Cefquinome disk; Oxoid, Basingstoke, Hampshire, England). The isolates were categorized as sensitive (zone diameter >20 mm), intermediate (zone diameter 16-20 mm) or resistant (zone diameter <16 mm) according to guidelines of the manufacturer. The minimum inhibitory concentration (MIC) was not measured.

Mammary quarter foremilk samples were collected for microbiological evaluation at 14 and 28 d after the last treatment. Bacteriological cure was defined as a treated infected mammary quarter that was bacteriologically negative for the presence of previously identified bacteria at 14 and 28 d after the last treatments. The relationship between the results of *in vitro* sensitivity test and bacteriological cure was examined using Mantel-Haenszel Chi-Square statistics with PROC FREQ statement of SAS (SAS version 8.2, SAS Inst., Inc., Cary, NC). *P*-values of <0.05 were considered to be significant.

Results

Most intramammary infections were due to Coagulase-Negative Staphylococci (CNS) (47.70%; 52/109), environmental Streptococci (18.35%; 20/109), and coliforms (15.60%; 17/109). One out of 110 quarters was excluded from this experiment because the susceptibility data were not available for the pathogens isolated from that quarter. The distribution of pathogens that caused subclinical intramammary infections across the treatment groups is presented in Table 1. The overall bacteriological cure rate for all intramammary infections was 84.61% (44/52) and 91.37% (53/58) for the standard and the extended regimen groups, respectively. A spontaneous cure rate of 20% was achieved in the negative untreated control group (further details concerning the untreated control group are not mentioned due to their irrelevance to the subject and aims of this paper).

The results of *in vitro* antimicrobial susceptibility testing for different pathogen groups are presented in Table 2. The bacteriological cure rates for sensitive, intermediate and resistant isolates in the standard treatment group were 82.4%, 90%, and 87.5%, respectively. These figures in the extended treatment group were 83.3%, 100%, and 100%, respectively. Treatment outcomes were not associated with the sensitivity test results in the standard group ($X^2 = 0.26$; *P*-value = 0.61). However, in the extended group, the probability of bacteriological cure was lower in quarters from which cefquinome-sensitive pathogens were isolated than the quarters from which intermediate or resistant pathogens were isolated. ($X^2 = 4.1$; *P*-value = 0.04; Table 3).

Discussion

Cefquinome is an advanced broad-spectrum cephalosporin with improved antibacterial activity over second and third generation cephalosporins (Sader and Jones, 1993). In the present study, treatment outcomes were not associated with the sensitivity test results in the standard group. Most previous studies on clinical and/or subclinical mastitis failed to demonstrate statistically significant correlations between the results of susceptibility testing and treatment outcomes for IMM pirlimycin (Cattell *et al*., 2001; Hoe and Ruegg, 2005; Apparao *et al*., 2009a), IMM penicillin-novobiocin (Owens *et al*., 1997), IMM cephapirin (Apparao *et al*., 2009b), and systemic oxytetracycline or systemic oxytetracycline plus IMM cephapirin (Constable and Morin, 2002). However, with ⩽58 infected quarters per group, the power of the

Table 1: Distribution of pathogens causing subclinical intramammary infections across the treatment groups.

Pathogen	Treatment groups*		Total
	Standard	Extended	
CNS[a]	27	26	53
C. bovis[b]	1	0	1
Environmental streptococci[c]	13	7	20
Coliforms[d]	7	10	17
S. aureus[e]	3	7	10
Others	1	8	9
Total	52	58	110

*Details concerning the untreated control group are not presented due to their irrelevance to the subject and aim of this paper.

[a]CNS: Coagulase-negative staphylococci

[b]*C. bovis: Corynebacterium bovis*

[c]Includes *Streptococcus dysgalactiae* subsp. *dysgalactiae* (predominant spp.) and *Streptococcus equinus.*

[d]Includes *E. coli* (predominant spp.), *Enterobacter aerogenes,* and *Klebsiella pneumonia.*

[e]*S. aureus: Staphylococcus aureus.*

Table 2: Qualitative results of in vitro antibiotic susceptibility testing for different pathogen groups.

Pathogen	Proportion of isolates in different susceptibility categories		
	Sensitive	Intermediate	Resistant
CNS	59.61% (31/52)	17.30% (9/52)	23.08% (12/52)
C. bovis	100% (1/1)	-	-
Environmental streptococci	50% (10/20)	30% (6/20)	20% (4/20)
Coliforms	58.82% (10/17)	11.76% (2/17)	29.41% (5/17)
S. aureus	100% (10/10)	-	-
Others	22.22% (2/9)	44.44% (4/9)	33.33% (3/9)

Table 3: The correlation of bacteriological test outcomes with the results of antibiotic susceptibility testing.

Cefquinome treatment group	Susceptibility category	Cure	Failure	*P*-value (one-sided)
Standard	Sensitive	82.4% (28/34)	17.6% (6/34)	0.61
	Intermediate	90% (9/10)	10% (1/10)	
	Resistant	87.5% (7/8)	12.5% (1/8)	
Extended	Sensitive	83.3% (25/30)	16.7% (5/30)	0.04
	Intermediate	100% (11/11)	0	
	Resistant	100% (16/16)	0	

present study to detect significant differences between treatment outcomes and results of sensitivity testing was limited. The failure to achieve a statistical power of 80% (β or type II error = 0.20) has also been a limitation in the majority of the above-mentioned studies.

Contrary to the results of the study reported here, some studies have demonstrated a significant positive correlation between the antimicrobial susceptibility testing and treatment outcomes for mild Gram-positive clinical mastitis treated with IMM cephapirin (Constable and Morin, 2002), and coliform mastitis treated with systemic trimethoprim-sulfonamide with or without non-steroidal anti-inflammatory agents (Shpigel *et al*., 1998). An apparent, but not statistically proven, association between the results of antimicrobial susceptibility tests and therapeutic outcomes were also found for short-term *S. aureus*, CNS, and streptococcal mastitis treated with IMM penicillin-novobiocin (Owens *et al*., 1997), as well as for environmental streptococcal mastitis treated with IMM pirlimycin (Cattell *et al*., 2001).

In the present study, in the extended group, the probability of bacteriological cure was lower in quarters from which cefquinome-sensitive pathogens were isolated than the quarters from which intermediate or resistant pathogens were isolated. The reason for this finding is unidentified but could be due to the small sample size in the intermediate and resistant categories.

The *in vitro* sensitivity despite "*in vivo* resistance" in the present study could be attributed to several factors: (1) the lack of *in vitro* susceptibility breakpoint data specific for mastitis in cows. With the exception of pirlimycin, ceftiofur, and penicillin-novobiocin combination, most breakpoints used to categorize mastitis pathogens as sensitive or resistant are derived from data on human pathogens and based on the pharmacokinetics of drugs in human serum (Apparao *et al*., 2009a, Constable *et al*., 2003). Therefore, zone diameters in the Kirby-Bauer test have not been related to antimicrobial concentrations achieved in the bovine mammary tissue for most antimicrobials (Constable *et al*., 2003). Several previous studies have failed to detect an association between the results of susceptibility testing and treatment outcome with regards to the use of pirlimycin for which validated breakpoints for bovine mastitis are available (Cattell *et al*., 2001; Hoe and Ruegg, 2005; Apparao *et al*., 2009a); (2) milk components in the udder (casein, calcium, lipids, and indigenous antibacterial agents) could potentially decrease the activity of many antimicrobials (Fang *et al*., 1996); (3) pharmacodynamic data concerning the IMM administration of antimicrobials in mastitic cows are limited (Constable *et al*., 2003); (4) antimicrobials could have detrimental effects on mammary defense mechanisms (Constable *et al*., 2003); (5) milking frequency (three times daily versus twice daily) may influence the concentration of antimicrobials at the site of infection in the mammary tissue, although in the case of cefquinome, the influence of individual cows has been more pronounced than that of milking frequency (Knappstein *et al*., 2003); and (6) mastitis pathogens in the test media multiply rapidly and are sensitive to antimicrobials, whereas these pathogens may have reduced multiplication rates in mastitic milk (Fang *et al*., 1996).

The reason for *in vitro* resistance despite apparent "*in vivo* susceptibility" is most likely to be related to the role of the mammary defense mechanisms in self-curing intramammary infections caused by several mastitis pathogens, particularly in CNS (Apparao *et al*., 2009a). However, this could not be the case in our study as, in contrast to the results of the study conducted by Apparao *et al*. (2009a), we found a significantly higher

bacteriological cure rate in the treated groups compared with controls (Kasravi *et al*., submitted). Additionally, a much lower spontaneous cure rate was found in our study (20%) versus the above-mentioned study (66%). The lack of validation of *in vitro* test relative to concentration and time above MIC of the antimicrobial at the actual site of infection in the mammary tissue could be another reason for *in vitro* resistance but *in vivo* susceptibility (Constable *et al*., 2003, Ruegg P. L., personal communication). Some researchers believe that for decision making in mastitis therapy, it is more informative for practitioners to know the causative pathogen rather than have results of the susceptibility test (Ruegg, P.L., personal communication). Interestingly, some researchers suggest that the results of susceptibility testing could be useful as a tool to understand the herd mastitis outbreaks caused by environmental pathogens; since similar sensitivity patterns among isolates could be interpreted as single strain predominance in these situations (Cattell *et al*., 2001). Furthermore, susceptibility testing can be useful for developing a "herd profile" of contagious mastitis pathogens, particularly in the case of *S. aureus*, to guide future treatment decisions (Hinckley *et al*., 1985).

The results of the present study indicate that Kirby-Bauer antimicrobial susceptibility testing does not predict bacteriological outcome in cows with persistent subclinical mastitis treated with IMM cefquinome. Further research is needed to elucidate the role, if any, that antimicrobial sensitivity testing should play in the treatment of clinical and/or subclinical mastitis in dairy cows.

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