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Relationship between *in vitro* antimicrobial sensitivity of bovine subclinical mastitis isolates and treatment outcome in lactating dairy cows

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Summary

The objective of the present study was to determine whether there was an association between the *in vitro* antimicrobial sensitivity test results of subclinical mastitis pathogens and bacteriological cure following intramammary treatment using a combination of nafcillin, penicillin, and streptomycin (NPS). Eighty-six intramammary pathogens from 43 cows were examined in this study. Most intramammary infections were due to coagulase-negative staphylococci, coliforms, and environmental streptococci. The antibiotic sensitivity to NPS was determined using the Kirby-Bauer disc diffusion method. Bacteriological cure rates for sensitive, intermediate, and resistant isolates in the standard treatment group (3 intramammary infusions of NPS once daily) were 84.4, 88.9, and 100%, respectively. These figures in the extended treatment group (6 intramammary infusions of NPS once daily) were 100% for the 3 categories. Bacteriological cure was not associated with the sensitivity test result. Based on this study, Kirby-Bauer sensitivity test results were not useful as predictors of the bacteriological outcome of subclinical mastitis treated with intramammary NPS.

Key words: Dairy cows, Subclinical mastitis, Sensitivity test, NPS treatment

Introduction

Antibacterial therapy is an important part of a mastitis control program in dairy cattle (Erskine and Wagner, 2003). *In vitro* antimicrobial sensitivity test of clinical or subclinical mastitis pathogens are frequently used by bovine practitioners to guide cow or herd level treatment decisions. However, for certain antimicrobial agents, previous studies failed to demonstrate statistically significant associations between the results of susceptibility testing and the 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*., 2009a, b). Previously, the *in vitro* and *in vivo* activity of the combination of nafcillin, penicillin, and streptomycin have been shown against a variety of mastitis pathogens, especially staphylococci (Phillips, 1979; Ziv *et al*., 1981; Bolourchi *et al*., 1995; Shpigel *et al*., 2006; Sampimon *et al*., 2007). The objective of the present study was to determine whether there was an association between the *in vitro* antimicrobial sensitivity test results of subclinical mastitis pathogens and the bacteriological cure of the intramammary (IMM) treatment using a combination of nafcillin, penicillin, and streptomycin (NPS).

Materials and Methods

The study was conducted in a closed, commercial, large Holstein dairy in Tehran province of Iran with an average of 1100 lactating dairy cows in summer, 2007. All lactating cows were milked thrice daily. The herd had a relatively low prevalence of *Staphylococcus aureus* and was free of

Streptococcus agalactiae and *Mycoplasma bovis* intramammary infections (IMI) based on several individual and bulk tank milk cultures and serological study. Mastitis pathogens were obtained from a randomized controlled clinical trial which was primarily aimed to evaluate the efficacy of standard and extended NPS IMM therapy for the treatment of persistent subclinical mastitis in lactating dairy cows in various stages of lactation. Briefly, mammary quarter foremilk samples from cows with composite milk SCC≥150,000/mL (based on monthly recording data, Animal Breeding Center of Iran) were obtained for microbiological and somatic cell count (SCC) analyses on 2 occasions 7 days apart. Forty three dairy cows with 86 infected quarters were enrolled in the study based on composite milk SCC≥150,000/mL on the last test day record, positive California mastitis test (CMT) results at the time of first pre-treatment sampling (scores 1, 2, and 3), quarter milk SCC≥200,000/mL and isolation of the same mastitis pathogen in the 2 samples obtained 7 days apart. CMT was used as a cow side screening test to prevent quarters with low scores from entering the experiment before knowing the SCC and culture results. All samples were collected immediately before routine milking. Sampling and microbiological procedures were conducted in accordance with the national mastitis council (NMC) standards (Oliver *et al*., 2004). Speciation of CNS, coliforms, streptococci and corynebacteria was made using biochemical tests specific for each organism as described by Quinn *et al*. (1994, 2002).

The infected quarters of the cows enrolled in the study were treated by IMM infusion of a commercial preparation consisting of 100 mg of sodium nafcillin plus 180 mg of sodium penicillin, and 100 mg of dihydrostreptomycin sulphate (Nafpenzal MC; Intervet International, Boxmeer, The Netherlands) every 24 h for 3 consecutive days (standard regimen group: 20 infected cows, 43 IMI), or every 24 h for 6 consecutive days (extended regimen group: 23 infected cows, 43 IMI). A negative untreated control group was also included.

Mueller-Hinton medium (Merck, Darmstadt, Germany) was used for the sensitivity testing of Gram-negative bacteria and staphylococci, and Mueller-Hinton medium with 5% defibrinated sheep blood was used for sensitivity testing of streptococci and corynebacteria. The mastitis isolates were evaluated for antimicrobial sensitivity via the Kirby-Bauer disc diffusion method. The procedure was performed in accordance with CLSI guidelines (Clinical Laboratory Standards Institute, 2008). Disks impregnated with 35 µg of nafcillin-penicillin-streptomycin combination were used to determine the susceptibility pattern (Nafpenzal disk, Oxoid, Basingstoke, Hampshire, England). The isolates were categorized as sensitive (inhibitory zone diameter >20 mm), intermediate (zone diameter 16-20 mm) or resistant (zone diameter <16 mm) according to the manufacturer's recommendation.

Mammary quarter foremilk samples were collected 14 and 28 days after the last treatment for microbiological culture. Bacteriological cures were defined as infections that were negative for the presence of previously identified bacteria at both 14 and 28 days after the last treatment. The relationship between the results of the *in vitro* sensitivity test and bacteriological cure was examined using Mantel-Haenszel Chi-square statistics with the PROC FREQ statement of SAS (2001). Values of $P<0.05$ were considered significant.

Results

Most IMI were due to coagulase-negative staphylococci [CNS] (58.14%: 50/86), coliforms (15.11%: 13/86), and environmental streptococci (11.63%: 10/86). The distribution of pathogens causing subclinical IMI across the treatment groups is presented in Table 1. The overall bacteriological cure rate for all IMI was 86.04% (37/43) and 100% (43/43) for the standard and the extended treatment groups, respectively. A spontaneous cure rate of 20% was achieved in the negative untreated control group. The results of the *in vitro* antimicrobial sensitivity 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 84.4, 88.9, and 100%, respectively. These figures in the extended treatment group were 100% for all susceptibility categories. Treatment outcomes were not associated with the sensitivity test results in the standard group ($X^2 = 0.41$; P-value = 0.52) (Table 3).

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

Pathogen	Treatment groups		Total
	Standard	Extended	
CNS[a]	21	29	50
C. bovis[b]	5	1	6
Environmental streptococci[c]	8	2	10
Coliforms[d]	6	7	13
S. aureus[e]	2	2	4
Others	1	2	3
Total	43	43	86

[a]CNS: Coagulase-negative staphylococci (*S. hyicus*, *S. auricularis*, *S. kloosi*, *S. hominis*, *S. muscae*, *S. carmosus*, *S. saprophyticus*, *S. epidermidis*, *S. milleri*, *S. caseolyticus*, and *S. sciuri*). [b]*C. bovis*: *Corynebacterium bovis*. [c]*Streptococcus dysgalactiae* (predominant spp.) and *Streptococcus equinus*. [d]*E. coli* (predominant spp.), *Enterobacter aerogenes*, and *Klebsiella pneumoniae*. [e]*S. aureus*: *Staphylococcus aureus*

Discussion

The objective of the present study was to determine whether there was an association between the *in vitro* antimicrobial sensitivity test results of subclinical mastitis pathogens and the bacteriological outcome of the IMM treatment using NPS.

In the study reported here, most IMI were due to CNS with an overall 78% *in vitro* sensitivity to NPS. It is numerically lower than that recently reported by Sampimon *et al*. (2007). The reason for this difference may lie in the low frequency of *S. chromogenes*, *S. xylosus*, and *S. simulans* in our study in contrast to the above mentioned study (Table 1).

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 associations 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*., 2009a), and systemic oxytetracycline or systemic oxytetracycline plus IMM cephapirin (Constable and Morin, 2002). However, with ≤43 infected quarters per group, the power of the present study to detect significant differences between the treatment outcomes and the results of sensitivity testing was limited. 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 abovementioned studies.

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

Pathogen	Proportion of isolates in different susceptibility categories		
	Sensitive	Intermediate	Resistant
CNS	78% (39/50)	16% (8/50)	6% (3/50)
C. bovis	83.3% (5/6)	0% (0/6)	16.7% (1/6)
Environmental streptococci	50% (5/10)	40% (4/10)	10% (1/10)
Coliforms	76.9% (10/13)	15.4% (2/13)	7.7% (1/13)
S. aureus	100% (4/4)	0% (0/4)	0% (0/4)
Others	100% (3/3)	0% (0/3)	0% (0/3)

Table 3: Bacteriological outcomes with the results of antibiotic susceptibility testing

NPS treatment group	Susceptibility category	Cure	Failure	P-value (one-sided)
Standard	Sensitive	84.4% (27/32)	15.6% (5/32)	0.52
	Intermediate	88.9% (8/9)	11.1% (1/9)	
	Resistant	100% (2/2)	0	
Extended	Sensitive	100% (34/34)	0	
	Intermediate	100% (5/5)	0	
	Resistant	100% (4/4)	0	

Contrary to the results of the study reported here, some studies have demonstrated a significant positive association between the antimicrobial susceptibility testing and the 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 (not statistically proven) association between the results of antimicrobial susceptibility tests and therapeutic outcomes were also found for short-term *Staphylococcus 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). The *in vitro* sensitivity but *in vivo* resistance in the present study could be attributed to several factors: (1) lack of *in vitro* susceptibility breakpoint data specific for mastitis in cows. With the exception of pirlimycin, ceftiofur, and penicillin-novobiocin, most breakpoints used to categorize mastitis pathogens as sensitive or resistant are derived from data on human pathogens and on the pharmacokinetics of drugs in human serum (Constable and Morin, 2003; Apparao *et al*., 2009a). Therefore, inhibitory zone diameters in the Kirby-Bauer test have not been related to antibiotic concentrations achieved in the bovine mammary tissue for most antibiotics (Constable and Morin, 2003). However, several previous studies have failed to detect an association between the results of susceptibility testing and the treatment outcome regarding 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) can potentially decrease the activity of many antibiotics (Fang and Pyörälä, 1996), (3) pharmacodynamic data regarding the IMM administration of antibiotics in mastitic cows are limited (Constable and Morin, 2003), (4) antibiotics can have a detrimental effect on mammary defense mechanisms (Constable and Morin, 2003), (5) Milking frequency (3 versus 2) may influence the concentration of antibiotics at the site of infection in the mammary tissue, and (6) mastitis pathogens in test media multiply rapidly and are sensitive to antibiotics, whereas these pathogens may have reduced multiplication rates in mastitic milk (Fang and Pyörälä, 1996).

It is stated that the reason for the *in vitro* resistance but apparent *in vivo* susceptibility is most likely related to the role of the cow immune system in self-curing IMI caused by several mastitis pathogens, particularly 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 the controls (unpublished data). Additionally, a much lower spontaneous cure rate was found in our study (20%) versus the abovementioned study (66%). The lack of validation of the *in vitro* test relative to the concentration and time above MIC of the antibiotic at the actual site of infection in the mammary tissue is another reason for the *in vitro* resistance but *in vivo* susceptibility (Constable and Morin, 2003). Some researchers believe that for decision making in mastitis therapy, it is more informative for practitioners to know the causative pathogen rather than having the results of the susceptibility test. 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 to guide future treatment decisions (Hinckley *et al*., 1985).

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

mastitis in dairy cows.

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