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Prevalence of and Antibiotic Susceptibility of Coagulase-Negatine *Staphylococci* Isolated from Bovine Intramammary Infections in Mashhad, Iran

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Abstract: Bovine mastitis is a frequent cause of economic loss in dairy herds. The Staphylococci are the predominant pathogen in subclinical and chronic bovine mastitis. Coagulase-Negative *Staphylococci* (CNS) have been isolated from milk samples collected from cows with clinical and subclinical mastitis in several countries, cause tissue damage and decreases in milk production. The purpose of this study was to determine the prevalence and *in vitro* antibiotic susceptibility of CNS isolated from bovine mastitis in Iran to several antimicrobial agents used in the control of this disease. In this study, 300 bovine milk samples with Intramammary Infections (IMIs) were collected from dairy herds in Mashhad, Iran over one year study period. From a total of 300 milk samples collected, 67 (22.33%) were positive for coagulase-negative *staphylococci*. CNS strains were identified as 18 (26.86%) strains of *S. hyicus*, 14 (20.89%) strains of *S. chromogenes*, 15 (22.39%) strains of *S. epidermidis*, 10 (14.92%) strains of *S. haemolyticus*, 3 (4.48%) strains of *S. sciuri*, 4 (5.97%) strains of *S. simulans* and 3 (4.48%) strains of *S. xylosus*. The antibiotic susceptibility test results showed that all CNS strains were susceptible to gentamycin (100%), while they were resistant to penicillin (95.52%), oxacillin (73.14%), sulfamethoxazole/trimethoprim (28.36%), erythromycin (22.39%), tetracycline (23.88%), kanamycin (14.92%), amoxycillin+clavulanic acid (14.92%), oxytetracycline (16.42%) and enrofloxacin (7.46%). Results of this study showed that coagulase-negative *staphylococcus* is emerging as important minor pathogens and can be the cause of substantial economic losses. The high resistance to penicillin found in this study emphasizes the importance of the identification of coagulase-negative *staphylococcus* when mastitis is present.

Key words: Antimicrobial susceptibility, intramammary infection, mastitis, Coagulase-Negative *Staphylococci* (CNS)

INTRODUCTION

Bovine mastitis is a frequent cause of economic loss in dairy herds. The epidemiology of bovine Intramammary Infections (IMIs) has been characterized worldwide by an increase in the prevalence of Staphylococci. Staphylococci are present as major mastitis pathogens in the dairy industry worldwide and the efficacy of the antibiotic used is paramount for successful elimination of infection. (Bradley, 2002; Maiti, 2003; Sharma, 2004; Hurettin *et al*., 2005). Coagulase-Negative *Staphylococcus* (CNS) has been previously considered a minor pathogen of bovine mastitis; however, many studies have recently shown the importance of CNS infection in the bovine mammary gland.

Several studies indicated that coagulase-negative *staphylococcus* is the most frequently recovered isolate from mastitis samples, especially in first lactation and unbred heifers (Devriese and DeKeyser, 1980; McDonald and Anderson, 1981; Chaffer *et al*., 1998; Myllys *et al*., 1998; Pengov, 2001; Gentilini *et al*., 2002; Rajala-Schultz *et al*., 2004; Moroni *et al*., 2004), cause tissue damage (Timms and Schultz, 1987) and decreases in milk production (Zhang and Maddox, 2000; Younis *et al*., 2003). Recent studies (Sharma, 2007) have demonstrated that the distribution of coagulase-negative *staphylococci* may be indicative of specific management practices such as postmilking teat antisepsis. Thus, accurate identification of these organisms is needed for development of improved mastitis control methods and epidemiological studies.

Antimicrobial drugs are mainly used in dairy herds for the prophylaxis and treatment of udder infections (Owens *et al*., 1997; Thornsberry *et al*., 1997) but also for other infections (Radostits *et al*., 2000). Many papers have reported susceptibility patterns of different mastitis

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pathogens (De Oliveira *et al*., 2000; Gentilini *et al*., 2002; Rossito *et al*., 2002; Tenhagen *et al*., 2006; Rosech *et al*., 2006).

The purpose of this study, was to determine the prevalence and *in vitro* antibiotic susceptibility of CNS isolated from bovine IMI in Iran to several antimicrobial agents used in the control of this disease and hence their potential usefulness for IMI therapy in cows.

MATERIALS AND METHODS

Bacterial isolates: Milk samples (10 mL) were taken aseptically from all quarters of 300 bovine infected udders of some dairy industry farms of Mashhad, Iran. The presence of CNS was determined by culturing 0.01 mL of each sample on 5% bovine blood agar (Merck, 10886.0500) plates and incubated at 37°C for 24-48 h. A quarter was identified as infected when a single pathogenic bacterium was isolated and Somatic Cell Count (SCC) were increased above 200,000 mL^{-1}.

The microbial strains were presumptively identified on the basis of morphology, haemolysis pattern and Gram staining of the colonies and then colonies of each microbial strain were streaked on blood agar to obtain a pure culture. Gram positive cocci were tested for catalase and coagulase production. The CNS species were identified by biochemical tests and then stored at -70°C in a nutrient broth (Oxoid, CM0001) enriched with 15% glycerol (Koneman *et al*., 1997). CNS identified and stored for subsequent antimicrobial susceptibility tests were: *S. epidermidis* (10 strains), *S. hyicus* (18), *S. haemolyticus* (10), *S.xylosus* (3), *S. sciuri* (3), *S. simulans* (4) and *S. chromogenes* (14).

Antimicrobial susceptibility: The antibiotic susceptibility tests for CNS isolates from mastitis milk samples were carried out by disc diffusion as described by Kirby-Bauer *et al*. (1966), using discs of penicillin, (10 IU); oxacillin, (1 μg); amoxycillin+clavullanic acid (20 μg + 10 μg), enrofloxacin (5 μg), sulphamethoxazole + trimethoprim (23.75 μg + 1.25 μg); kanamycin, (30 μg); gentamicin, (10 μg); erythromycin, (15 μg); tetracycline, (30 μg) and oxytetracycline, (30 μg). Each isolate was first poured on Mueller Hinton agar (MH) (Merck, 5437.0500). Then antibiotic discs were placed on the MH agar plates and then the plates were incubated at 37°C for 24 h. The isolated bacteria were categorized as susceptible, intermediate and resistant based upon interpretive criteria developed by the National Committee of Clinical Laboratory Standards (NCCLS, 2002). *Staphylococcus aureus* ATCC 25923 was used as a control in all of the assays.

RESULTS AND DISCUSSION

From a total of 300 mastitis milk samples collected, 67 (22.33%) were positive for coagulase-negative *staphylococci*. CNS strains were identified as 18 (26.86%) strains of *S. hyicus*, 15 (22.39%) strains of *S. epidermidis*, 14 (20.89%) strains of *S. chromogenes*, 10 (14.92%) strains of *S. haemolyticus*, 3 (4.48%) strains of *S. sciuri*, 4 (5.97%) strains of *S. simulans* and 3 (4.48%) strains of *S. xylosus* (Table 1).

Coagulase-negative *staphylococci* are frequently isolated as causative agents of nosocomial infections, urinary tract infections from human patients and can also cause serious infections in humans, in particular endocarditis. Rajala-Schultz *et al*. (2004) reported that CNS is increasing its importance as cause of bovine mastitis throughout the world in recent years. CNS infections are associated with damage to milk secretory tissue of the mammary gland by increased connective tissue stroma, moderate increases in milk SCC and significant production decreases (Timms and Schultz, 1987; Chaffer *et al*., 1998; Chaffer *et al*., 1999; Oliver *et al*., 2003). The role of CNS as a possible reservoir for resistance among staphylococci has been determined (Archer and Climo, 1994). Bacterial identification and susceptibility tests are important for selecting the appropriate antimicrobial agent when treating bovine mastitis. The pathogenicity of the isolates, the growing incidence and the resistance to antimicrobial drugs have recently become the objects of many studies (Gentilini *et al*., 2002; Moroni *et al*., 2004). Myllys *et al*. (1994) reported the growing incidence of *Staphylococcus aureus* and CN *staphylococci* in milk and discussed probable reasons of a more rapid growth of CN staphylococci than of *S. aureus*. In their subsequent study (Myllys *et al*., 1998) CN *staphylococci* accounted for 53.5% and *S. aureus* for 16.7% of the total bacterial burden of milk. The high prevalence of CNS may be attributed to the wide distribution of the organism inside the mammary gland and in the skin of the teats and udder (McDonald and Anderson, 1981; Radostits *et al*., 2000). Chaffer *et al*. (1999) branded CN *staphylococci* the most frequently occurring bacteria in milk of cows affected by mastitis, which are able to stimulate the host's immune system and persist in infected mammary glands for long periods.

The *in vitro* activities of each of the antimicrobial agents tested against CNS are summarized in Table 2. All isolates (100%) were susceptible to gentamicin whereas 64 isolates (95.52%), 10 isolates (14.92%), 10 isolates (14.92%), 5 isolates (7.46%), 15 isolates (22.39%), 49 isolates (73.14%), 19 isolates (28.36%), 11 isolates (16.42%) and 16 isolates (23.88%) were resistant to

Table 1: Prevalence of Coagulase-Negative *Staphylococci* (CNS) isolated from bovine mastitis milk samples

CNS isolate	Number	(%)
S.hyicus	18	26.86
S.epidermidis	15	22.39
S.chromogenes	14	20.89
S.heamolyticus	10	14.92
S.sciuri	3	4.48
S.simulans	4	5.97
S.xylosus	3	4.48
Total	67	100

Table 2: Antibiotic susceptibility of 67 isolated coagulase-negative *Staphylococci* from bovine mastitis milk samples

Antibiotics	Resistance No (%)	Intermediate No (%)	Sensitive No (%)
Penicillin (10 IU)	64 (95.52%)	3 (4.48%)	0
Erythromycin (15 μg)	15 (22.39%)	4 (5.97%)	48 (71.64%)
Amoxycillin+Clavulanic acid (20+10 μg)	10 (14.92%)	3 (4.48%)	54 (80.6%)
Enrofloxacin (5 μg)	5 (7.46%)	2 (2.98%)	60 (89.55%)
Kanamycin (30 μg)	10 (14.92%)	4 (5.97%)	53 (79.1%)
Gentamycin (10 μg)	0	0	67 (100%)
Oxacillin (1 μg)	49 (73.14%)	5 (7.46%)	13 (19.4%)
Sulfamethoxazole/ Trimethoprim (23.75+1.25 μg)	19 (28.36%)	4 (5.97%)	44 (65.67%)
Tetracycline (30 μg)	16 (23.88%)	3 (4.48%)	48 (71.64%)
Oxytetracycline (30 μg)	11 (16.42%)	4 (5.97%)	52 (77.61%)

penicillin, amoxycillin + clavulanic acid, kanamycin, enrofloxacin, erythromycin, oxacillin, sulfamethoxazole/ trimethoprim, oxytetracyclin and tetracycline, respectively.

Susceptibility of CNS isolated from bovine mastitis to selected antimicrobial agents has been previously reported (McDonald and Anderson, 1981; Frigerio *et al*., 1995; Thornsberry *et al*., 1997; Myllys *et al*., 1998; Gentilini *et al*., 2002; Rossitto *et al*., 2002; Guerin-Faublee *et al*., 2003). In many studies, evaluating susceptibility patterns of mastitis pathogens, CNS have been reported to show most resistance against penicillin (Myllys *et al*., 1998; Gentilini *et al*., 2002). Gentilini *et al*. (2002) reported that coagulase-negative *staphylococci* exhibited the highest degree of resistance to penicillin of all antimicrobial agents. Penicillin-resistance found in our study was higher than previous studies reported for Argentina (30%; Frigerio *et al*., 1995), Finland (37.2% and 32%; Myllys *et al*., 1998; Pitkala *et al*., 2004) and the United States (42.7 and 57%; McDonald and Anderson, 1981; Owens *et al*., 1997). Archer and Scott (1991) reported that an inducible β-lactamase was present in 80 to 90% of human CNS isolates. Other authors reported that β-lactamase was produced by 34.1% (Myllys *et al*., 1998) and 83.6% (Thornsberry *et al*., 1997) of the CNS isolates derived from bovine mastitis.

Gentamicin was very active against CNS, as all tested isolates were susceptible. Similar results were found in Argentina (Frigerio *et al*., 1995) and in the United States (McDonald and Anderson, 1981) for gentamicin and cephalothin.

The resistance rate of CNS against erythromycin reported in this study (22.39%) was higher than 11.5% (Myllys *et al*., 1998), 10% (Owens *et al*., 1997) 1.5% (Frigerio *et al*., 1995) and the United States (McDonald and Anderson, 1981) found in previous studies. Erythromycin, oxytetracycline, tetracycline and sulfamethoxazole-trimethoprim showed moderate activity against the CNS tested.

The emergence and dissemination of antimicrobial resistance is the result of numerous complex interactions among antimicrobials, microorganisms and the surrounding environment (O'Brien, 2002). The two main factors involved in the development of antibiotic resistance in bacteria are the selective pressure by the use of antibiotics and the presence of resistance genes (Witte, 2000). The observation of CNS exhibiting most resistance against penicillin appears to be in agreement with the antibiotic usage patterns in the dairy farms. Penicillin is used post operatively after surgical correction of abomasal displacements and also for foot rot. There is growing evidence and little doubt that resistance genes can be spread and exchanged between different bacterial populations (O'Brien, 2002).

CONCLUSION

Results of this study, showed that coagulase negative *staphylococcus* is emerging as important minor mastitis pathogens and can be the cause of substantial economic losses. The high resistance to penicillin and oxacillin found in this study emphasize the importance of identification of CNS when an intramammary infection is present. Antimicrobial susceptibility patterns should be identified for CNS, as current susceptibility data are necessary to select appropriate antibiotics for a successful treatment. Further, studies are necessary for assessing the importance of mastitis caused by CNS in Iran. CNS resistance in dairy herds in Iran must be monitored continuously in order to develop susceptibility patterns and to establish trends in CNS resistance and appropriate drycow therapy.

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