

Characteristics of staphylococci isolated from clinical and subclinical mastitis cows in Shahrekord, Iran

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Summary

Staphylococci isolated from 98 mastitic cow's milk samples were analyzed for the production of alpha, beta and delta-hemolysin on sheep, bovine and horse red blood cells and *in vitro* susceptibility to several antimicrobial agents. Out of a total of 18 isolated strains, 100% demonstrated hemolytic activity either alone or in combined forms. Among 8 isolates of *S. aureus* 3 isolates produced α/β and 5 produced $\alpha/\beta/\delta$ hemolysins. From 10 isolates of coagulase-negative staphylococci (CNS), 2 α , 2 δ , 2 α/δ and 4 $\alpha/\beta/\delta$ hemolysins producing isolates were confirmed. *S. aureus* isolates were resistant to cloxacillin (100%), penicillin (87%) and ampicillin (62.5%). CNSs showed resistance only to amoxicillin (60%). The results suggest that a cow's infected mammary glands are an important reservoir of hemolytic staphylococci. A high degree of *S. aureus* antimicrobial resistances was detected.

Key words: Cow, Mastitis, Staphylococci, Hemolysin, Antimicrobial susceptibilities

Introduction

Staphylococcal strains from animals have not been studied as extensively as strains associated with human infections. These strains are considered important etiological agents involved in all forms of cow mastitis (Eberhart *et al.*, 1987). The pathogenicity of the genus *Staphylococcus*, particularly for *S. aureus*, is related to the production of a wide variety of exoproteins, including the alpha, beta, and delta-hemolysins which contribute to its ability to cause diseases in many mammalian species (Dinges *et al.*, 2000). The role of *Staphylococcus aureus* and coagulase-negative staphylococcal exosecretions in bovine udder infection was the subject of some recent studies (Fitzgerald *et al.*, 2000; Younis *et al.*, 2003; da Silva *et al.*, 2005). Staphylococcal alpha-hemolysin or alpha-toxin is the most studied and characterized cytotoxin, and is considered a main pathogenicity factor because of its hemolytic, dermonecrotic and neurotoxic effects (Dinges *et al.*, 2000). Additionally, beta-hemolysin is a sphingomyelinase that is

highly active against sheep and bovine erythrocytes (Larsen *et al.*, 2002), while delta-hemolysin as well as alpha-hemolysin induce pore formation, perturbing the cell membrane permeability (Butt *et al.*, 1998).

The public health significance of staphylococci isolated from milk and dairy products is important. It has been suggested that cattle can be a source of antibiotic-resistant strains for humans (Thatcher and Simon, 1955).

On the other hand, identification of mastitis pathogens and their antimicrobial susceptibilities is important when selecting appropriate antimicrobial therapy. The present study was designed to determine the species, hemolytic ability and antimicrobial susceptibilities of the staphylococci isolated from clinical and subclinical cow mastitis in Shahrekord dairy herds in western areas of central Iran.

Materials and Methods

Sample collection

Fifty, 50 and 48 milk samples were obtained from normal, subclinical and

clinical mastitis cows, respectively. Taking normal samples was designed to exclude the possibility of considering normal bacterial flora as causative agents of mastitis. Mastitis was diagnosed on the basis of California mastitis test (CMT), clinical examination of the udder and macroscopic evaluation of secretions.

The study covered 20 months from March 2006 to October 2007. Normal cows were defined as CMT negative, and the absence of any clinical signs of mastitis; subclinical mastitis was CMT positive (2 + or more) in the absence of clinical signs, and clinical mastitis cows were those that had clinical signs (Esron *et al.*, 2005). Milk samples were aseptically collected from each quarter. Briefly, immediately after the pre-milk hand-stripping, teat orifices were scrubbed with a cotton pledget saturated in 70% ethyl alcohol. The ice box containing milk samples in tubes was sent to the microbiology lab of the Veterinary College of Shahrekord University to isolate the strains. On arrival in the laboratory, 0.1 ml of milk was streaked onto 5% sheep blood agar plates using a sterile cotton swab and incubated at 37°C for 18 to 24 h. The presence of more than 3 colonies of a similar morphotype was accepted as a positive bacteriological finding (Deinhofer and Pernthaner, 1995).

Colony morphology, gram staining and catalase test were used to identify bacterial genus and were followed by further biochemical tests.

Identification of *Staphylococcus* species

The species identification was carried out according to Ballows *et al.* (1985). The colonies identified as *Staphylococcus* were submitted to coagulase test using rabbit plasma (Quinn *et al.*, 1994). The catalase and oxidase tests were followed by examining the susceptibility profile of isolates towards bacitracin. The tests of sucrose, D-mannose, D-manitol, maltose, D-trehalose, raffinose fermentation, urease activity and acetoin production were also assayed. All tests were performed as described by Quinn *et al.* (1994). The isolates were kept frozen at -70°C in

Tryptose soy broth containing 15% (v/v) glycerol until the hemolytic tests were carried out.

Essay for hemolytic activity

The hemolytic activity was evaluated by plating staphylococci strains on triplicate plates of blood agar base supplemented with 5% sheep, bovine and horse blood for alpha, beta and delta-hemolysin assays, respectively (Quinn *et al.*, 1994). To remove any possible anti-hemolysin compounds present in the serum, the red blood cells were washed with sterile saline and resuspended in saline to the original volume of the blood. Strains were inoculated in the form of streaks on the surface of plates and incubated at 37°C for 24 and 48 h. The criteria for hemolysin identification were: complete lytic zone (transparent) with blurred edges for alpha-hemolysin on ovine and incomplete (non-transparent) lytic zone, which became complete with sharp edges after overnight incubation at 4°C on bovine blood agar, for beta-hemolysin. The delta-hemolysin production was determined as complete hemolytic zones on horse blood agar (Quinn *et al.*, 1994).

Susceptibility testing

For susceptibility testing, isolates were incubated in trypticase soy broth at 37°C for 24 h and the suspension was adjusted to a turbidity equivalent to a 0.5 McFarland standard. Susceptibility to antimicrobial agents was determined for isolated strains by the disk diffusion method on Mueller-Hinton agar following the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 2002). The selected antibiotics for antibiogram were cephalexine, penicillin, clindamycin, cloxaciline, gentamicin, streptomycin, amoxicillin, tetracycline, kanamycin, oxytetracycline, ampicillin, chloramphenicol and erythromycin that were more common in the treatment of the regional bovine mastitis cases.

Isolates were categorized as susceptible and resistant based upon interpretive criteria developed by the National Committee for Clinical Laboratory Standards (NCCLS, 1999).

Results

A total of 18 (18.36% of samples) gram-positive, catalase-positive strains of cocci (2 from clinical and 16 from subclinical mastitis cases), were isolated. Such cocci was not isolated from normal milk samples. Overall five species were identified.

From subclinical mastitis, 8 strains of *Staphylococcus aureus* were confirmed and found to be the most common (44.44%) followed by 4 strains of *Staphylococcus hyicus* (22.22%), 2 of *Staphylococcus epidermis* (11.11%), one of *Staphylococcus intermedius* (5.55%) and one *Staphylococcus captis* (5.55%). The isolates from clinical mastitis were 2 isolates of *Staphylococcus hyicus*, representing 11.11% of all 18 strains.

Hemolytic activity was found in 100% of the total 18 strains studied. Fifty percent (4 out of 8 strains) of coagulase-negative staphylococci from subclinical mastitis produced hemolysis caused by at least one type of hemolysin and among these 8 isolated strains, 2 strains were producers of alpha-hemolysin, 2 delta and 3 were $\alpha/\beta/\delta$ hemolysins producers. Out of 8 *S. aureus* isolates 5 strains produced $\alpha/\beta/\delta$ and 3 produced α/β hemolysins. In the clinical mastitis samples only 2 isolates of *S. hyicus*, one producing $\alpha/\beta/\delta$ and the other α/δ hemolysins were detected.

The antimicrobial susceptibility results for isolates of *S. aureus* and coagulase

negative staphylococci are summarized in Table 1. *S. aureus* isolates were resistant to cloxacillin (100%), penicillin (87%) and ampicillin (62.5%). Coagulase negative staphylococci showed resistance only to amoxicillin (60%).

Discussion

Diseases caused by staphylococci are the result of a synthesis of several virulence factors including the different hemolysins, which are important for virulence of the *S. aureus* and other species.

In this study, 10 CNSs isolates out of 18 isolations were detected belonging to 10 out of the 98 tested samples. This is in accordance with Gentilini *et al.* (2002) who examined 123 mastitis cow milk samples in Argentina and found 13.6% were positive for CNS. In general, the role of CNS as a cause of bovine mastitis is not completely clear. Jorun (1991) reported that distribution of CNS species in milk samples was similar regardless of whether the cow suffered from clinical or subclinical mastitis.

All of the isolated CNS demonstrated hemolytic activity either alone or in combined forms. Isolated *S. aureus* strains also produced $\alpha/\beta/\delta$ or α/β hemolysins in all 8 isolations. A similar study in mastitis goat milk in Brazilian dairy herds showed high levels of single or combined hemolysin types produced by *S. aureus* and CNS (da Silva *et al.*, 2005). A possible involvement

Table 1: Antibiotic susceptibility responses of staphylococci isolated from mastitis cow milk samples

Antibiotics	Staphylococci			
	<i>Staphylococcus aureus</i>		<i>Coagulase negative staphylococci</i>	
	S (%)	NS (%)	S (%)	NS (%)
Cephalexine	8 (100%)	0 (0.0%)	10 (100%)	0 (0.0%)
Penicillin	1 (12.5%)	7 (87.5%)	9 (90%)	1 (10%)
Clindamycin	7 (87.5%)	1 (12.5%)	10 (100%)	0 (0.0%)
Cloxaciline	0 (0.0%)	8 (100%)	10 (100%)	0 (0.0%)
Gentamicin	8 (100%)	0 (0.0%)	10 (100%)	0 (0.0%)
Streptomycin	7 (87.5%)	1 (12.5%)	10 (100%)	0 (0.0%)
Amoxicillin	8 (100%)	0 (0.0%)	4 (40%)	6 (60%)
Tetracycline	8 (100%)	0 (0.0%)	10 (100%)	0 (0.0%)
Kanamycin	7 (87.5%)	1 (12.5%)	9 (90%)	1 (10%)
Oxytetracycline	8 (100%)	0 (0.0%)	9 (90%)	1 (10%)
Ampicillin	3 (37.5%)	5 (62.5%)	8 (80%)	2 (20%)
Chloramphenicol	8 (100%)	0 (0.0%)	10 (100%)	0 (0.0%)
Erythromycin	7 (87.5%)	1 (12.5%)	9 (90%)	1 (10%)

S = Sensitive and NS = Non sensitive

of these hemolysins in different pathological processes was aroused by Butt *et al.* (1998) who reported hemolytic activity in 49% of CNS strains isolated from human chronic muscular disease.

In the present study all 6 isolates of *S. hyicus* produced single or combined types of hemolysins. There is evidence that *S. hyicus* is more pathogenic than the other species belonging to coagulase negative staphylococci (Jorun, 1991). Our results are in agreement with reports that indicate this species as the most frequently isolated CNS from milk with abnormal or high SCC (Devriese and De Keyser, 1980).

Out of 16 isolated staphylococci strains from subclinical mastitis samples, 4 (25%) produced single type (α or δ) and 12 (75%) produced combined hemolysins. While in both of the clinical mastitis isolated strains, combined $\alpha/\beta/\delta$ or α/δ hemolysins production were observed. Kenny *et al.* (1992) reported that the interaction of these toxins in mastitis cases might lead to clinical signs. However, as we had only two isolates from clinical mastitis samples, it is difficult to conclude any relation between the type of hemolysin production and the type of mastitis.

Eight (out of 16) isolated strains from the subclinical mastitis samples were *S. aureus*, which has been considered the major pathogen. Radostits *et al.* (2000) asserted that *S. aureus* is well adapted to survival in the udder and usually establishes a mild subclinical infection over a long duration. The data from this study suggest that cows infected mammary glands are an important source of hemolytic staphylococci.

The high resistance of *S. aureus* to cloxacillin, penicillin and ampicillin (100, 87 and 62.5%, respectively) found in this study emphasizes the importance of the identification of *S. aureus* when a subclinical mastitis is present. However, antimicrobial resistancy patterns of coagulase negative staphylococci were found only to amoxicillin (60%). Other studies show some resistance of CNS to penicillins, but a limited resistance to other antimicrobial agents (Werckenthin *et al.*, 2001).

In conclusion, the *S. aureus* and particularly the CNS abilities to produce

different hemolysins may contribute to the persistence of intramammary infection caused by these microorganisms. This study showed that coagulase negative staphylococci are emerging as important minor mastitis pathogens and can be the cause of substantial economic losses.

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