Characterization of hemolysins of *Staphylococcus* strains isolated from human and bovine, southern Iran

Moraveji, Z.[1]; Tabatabaei, M.[2*]; Shirzad Aski, H.[3] and Khoshbakht, R.[4]

[1]DVM Student, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [2]Department of Pathobiology, School of Veterinary Medicine and Institute of Biotechnology, Shiraz University, Shiraz, Iran; [3]Ph.D. Student in Bacteriology, Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [4]Graduated from School of Veterinary Medicine, Shiraz University, Shiraz, Iran

[*]***Correspondence:*** *M. Tabatabaei, Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. E-mail: mtabatabaei2003@yahoo.co.uk*

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Summary

The staphylococci are important pathogenic bacteria causing various infections in animals and human. Hemolysin is one of the virulence factors of coagulase-positive (CPS) and coagulase-negative staphylococci (CNS). The aims of the study were to characterize hemolysins of *Staphylococcus* spp. isolated from human and bovine origin, phenotypic- and genotypically. Characterization of hemolysin phenotypically based on hemolysis pattern of *Staphylococcus* spp. was done on the sheep, horse and rabbit blood agar plates. Genes encoding hemolysin were amplified with specific primers by using polymerase chain reaction (PCR) technique. Hemolytic activities phenotypically were determined in 60 and 90% of the total bovine and human isolates, respectively. All non hemolytic isolates were CNS ($P \leq 0.05$). In all isolates, *hla* and *hld* genes were determined by PCR amplification. None of the bovine and human isolates showed phenotypically and genotypically gamma hemolysin. The results from this study suggest that, in accordance with what is generally believed, some differences are apparent in hemolysin types among *Staphylococcus* strains of bovine and human origin. Furthermore, this study showed that CNS can be important as new pathogens.

Key words: *Staphylococcus* spp., Hemolysin, Human and bovine isolates, PCR

Introduction

The Staphylococci are well known as bacterial pathogens causing multiple types of infections in both human and animals (Le Loir *et al*., 2003; Salasia *et al*., 2004). In infection diagnostic, staphylococci are divided into coagulase-positive (CPS) and coagulase-negative staphylococci (CNS) based on the ability to coagulate rabbit plasma. Generally, *Staphylococcus aureus* is coagulase positive although coagulase-negative isolates of *S. aureus* do occur (Fox *et al*., 1996). Although *S. aureus* has been considered as pathogen, CNS are the most frequently isolated, especially from pyogenic infections in human and subclinical mastitis in cows, sheep and goats (Le Loir *et al*., 2003; Salasia *et al*., 2004; da Silva *et al*., 2005). Some species of this genus, particularly *S. aureus*, cause a variety of diseases by production of a series of enzymes and toxins, invasion of host cells like hemolysins (da Silva *et al*., 2005). At present, the hemolysins of staphylococci are classified in four different types including alpha (α), beta (β), gamma (γ) and delta (δ) (Aarestrup *et al*., 1999). Alpha toxin is a heptamer pore-forming exotoxin that lyses primarily rabbit erythrocytes but is toxic to human epithelial cells (Gouaux *et al*., 1994). Beta-hemolysin is a sphingomyelinase that is highly active against sheep and bovine erythrocytes (Larsen *et al*., 2002). Beta toxin is also known as the hot-cold toxin because of its unique activity on sheep blood agar plates. At 37°C, beta toxin interacts with sheep red blood cells but does not lyse them. If the red cells are then placed at 4°C, the cells lyse; this is observed as a lack of hemolysis on blood agar plates at 37°C and then complete hemolysis at 4°C (Huseby *et al*., 2007). Gamma toxin is a two-component exotoxin comprising at least six different combinations of proteins, one of which is leukocidin which affects the horse red blood cells (Dinges *et al*., 2000). Delta toxin is a low-molecular-weight exotoxin that forms multimeric structures with the ability to lyse many cell types (Novick *et al*., 2003). Several studies indicated that hemolysins of *S. aureus* correlated well with infections in human and animals (Tackeuchi *et al*., 2001; Larsen *et al*., 2002). However, there is scarce information about hemolysins production by CNS isolated from animal mastitis and human infections, especially in Iran. Genotypic differences between *S. aureus* isolated from human and bovine have been observed by numerous authors (Zadoks *et al*., 2000; Reinoso *et al*., 2004). One of the key characteristics used to distinguish human and bovine strains is the hemolytic pattern. Thus, most CPS bovine isolates have been reported to produce beta hemolysin and to be variable alpha hemolysin producers, whereas most CPS human isolates produce alpha hemolysin but only a limited number produce beta hemolysin (Hummel *et al*., 1992; Aarestrup *et al*., 1999). To date, little information has been reported about hemolytic pattern in CNS. So, the purpose of our present study was to determine and compare the occurrence of

hemolytic staphylococci isolated from bovine and human origin, by both phenotypic and genotypic methods.

In addition, we demonstrated hemolytic ability of the CNS isolated from animal mastitis and human infections in Shiraz, in the southern of Iran.

Materials and Methods

Bacterial isolates

A total number of 40 *Staphylococcus* spp. isolated from human infections (n=20) and cow mastitis (n=20) and also 9 reference strains (IROST) of *Staphylococcus* spp. were used in the present study. The samples of *Staphylococcus* spp. were obtained from skin infections of patients at Namazi Hospital and the mastitic milk of 20 dairy cattle from 15 farms located in Shiraz, in the Southern of Iran. First, different isolates were identified as CPS and CNS based on Gram staining, coagulase and catalase test, tellurite reduction, lecithinase activity and mannitol fermentation on mannitol salt agar (MSA). Furthermore, the isolates were identified by genus PCR amplification of the 16S rRNA gene (Data not shown).

Hemolysins characterization

Types of hemolysins were characterized based on the lysis zone of each staphylococcal isolate on triplicate plates of blood agar base supplemented with 5% sheep, horse and rabbit blood after 24 and 48 h incubation at 37°C. 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 (Ebrahimi *et al*., 2009). The genes encoding the hemolysins of *Staphylococcus* spp. were performed by amplification of *hla*, *hlb*, *hld* and *hlg* genes for alpha, beta, delta and gamma hemolysin using PCR method, respectively. The PCR programs and the sequences of the primers are listed in Table 1.

DNA preparation and PCR assay

The bacterial genomic DNA was extracted from overnight cultures of isolates by using the procedure described previously (Ahmadi *et al*., 2010). The purity and concentration of the DNA were estimated by spectrophotometry at 260 and 280 nm. After 16S rRNA gene PCR was done for identification of *Staphylococcus* spp., and for detection of hemolysin genes listed in Table 1 for each confirmed isolate. Amplification of bacterial DNA was performed in a total reaction volume of 25 µl which contained 2 µl of DNA template from pure cultures. The reaction mixture consisted of 2.5 µL 10 x PCR buffer (75 mM Tris-HCl, pH = 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $[NH_4]_2SO_4$), (CinnaGen, Iran), 1 µL dNTPs (50 µM), (CinnaGen), 1 µL (1 U Ampli Taq DNA polymerase), (CinnaGen), 1 µL (25 pmol) from the forward and reverse primers (CinnaGen), shown in Table 1, and the volumes of the reaction mixtures reached 25 µL using distilled deionized water. Template DNA was initially denatured at 94°C for 7 min. Subsequently, a total of 35 amplification cycles were carried out in a programmable thermocycler (MJ mini, BioRad, USA). Each cycle consisted of denaturation for 1 min at 94°C, primer annealing for 1 min at 58°C and extension for 1 min at 72°C. The last cycle was followed by a final extension at 72°C for 7 min and then the PCR products remained in the thermal cycler at 4°C until they were collected. Negative control containing water was included in each experiment. The PCR products were resolved by electrophoresis in a 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining and UV transillumination (BTS-20, Japan). The 100-bp DNA ladder (CinnaGen, Iran) was used as a molecular size marker (Fig. 1).

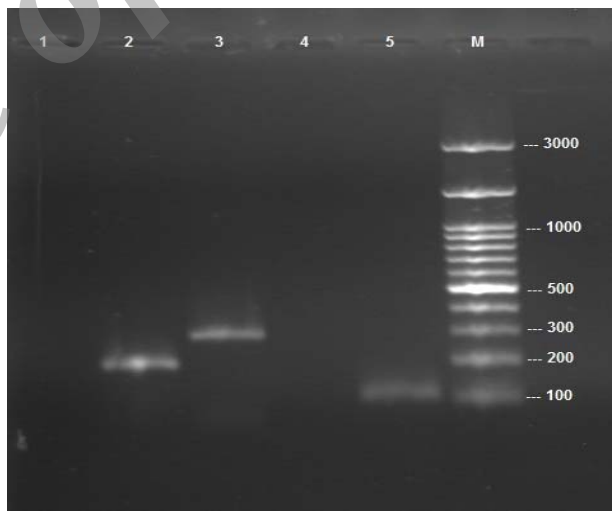

Fig. 1: Agarose gel electrophoresis of PCR products of the hemolysin genes of *Staphylococcus* spp. Lane M: The DNA 100 bp marker (CinnaGen, Iran). Lane 1: Negative sample, Lane 2: *hla* (209 bp), Lane 3: *hlb* (309 bp), Lane 4: *hlg* (535 bp) [no positive], and Lane 5: *hld* (111 bp)

Table 1: Nucleotide sequences used as primers for the detection of the hemolysin genes

Name of primer	Sequence (5′ to 3′)	Target gene	Annealing temperature (°C)	Product size (bp)	Reference
HLA-1	CTGATTACTATCCAAGAAATTCGATTG	*hla*	58	209	Jarraud *et al*., 2002
HLA-2	CTTTCCAGCCTACTTTTTTATCAGT				
HLB-1	GTGCACTTACTGACAATAGTGC	*hlb*	58	309	Jarraud *et al*., 2002
HLB-2	GTTGATGAGTAGCTACCTTCAGT				
HLD-1	AAGAATTTTTATCTTAATTAAGGAAGGAGTG	*hld*	58	111	Jarraud *et al*., 2002
HLD-2	TTAGTGAATTTGTTCACTGTGTCGA				
mpHLG-1	GTCAYAGAGTCCATAATGCATTTAA	*hlg*	58	535	Jarraud *et al*., 2002
mpHLG-2	CACCAAATGTATAGCCTAAAGTG				

Statistical analysis

For statistical analysis, a Chi-square test was performed to analyze the association of the hemolysin genes in human and bovine samples. A p-value of ≤0.05 was considered as statistically significant.

Results

According to the results of cultural and biochemical properties, along with amplification of the 16S rRNA, all isolates examined in the present investigation were identified as *Staphylococcus*. In total, 5 and 8 out of 20 bovine and human isolates were CPS, respectively. Phenotypic hemolysin activity was found in 60 and 90% of the total bovine and human isolates. The presence of non-hemolytic isolates was significantly different among CNS and CPS and all non hemolytic isolates belong to CNS (P≤0.05). On the sheep blood agar plates, most of the *Staphylococcus* isolated from bovines showed delta hemolysin while the most of the human isolates produced alpha hemolysin. Out of 20 human isolates 10 (50%) produced double hemolysin (DH) but bovine isolates showed significantly low DH ratio (1 out of 20) (P≤0.05). By PCR amplification of the gene encoding hemolysin of *Staphylococcus* spp. with specific primers it could be observed that *hla* and *hld* genes were present in all isolates. Also, in combination with *hla* and *hld*, the *hlb* gene was found in 3 (15%) out of the 20 *Staphylococcus* isolates collected from bovines, 8 (40%) out of the 20 isolates collected from humans that were significantly different (P≤0.05), and 4 (44.4%) out of the 9 reference strains. None of the human and bovine isolates showed phenotypically and genotypically gamma hemolysin. Distribution of the various hemolysin genes among the *Staphylococcus* spp. investigated in the present study is shown in Table 2.

Table 2: Characteristic of hemolysins of *Staphylococcus* strains

Strains	Coagulase test	Phenotypic characterization			Genotypic characterization
		Horse BA	Rabbit BA	Sheep BA	
S. saprophyticus PTCC 1440	-	NC	NC	NC	α, δ
S. xylosus PTCC 1444	-	NC	NC	β	α, β, δ
S. simulans PTCC 1442	-	δ	δ	β	α, β, δ
S. chromogenes PTCC 1433	-	NC	NC	NC	α, δ
S. intermedius PTCC 1438	+	α	α	β	α, β, δ
S. haemolyticus PTCC 1437	-	α	α	α	α, δ
S. epidermidis PTCC 1436	-	α	α	β	α, β, δ
S. sciuri PTCC 1441	-	δ	δ	δ	α, δ
S. aureus PTCC 1764	+	NC	α	α	α, δ
Bovine 1	-	NC	NC	NC	α, δ
Bovine 2	-	NC	NC	NC	α, δ
Bovine 3	-	δ	δ	δ	α, δ
Bovine 4	+	NC	α	β	α, β, δ
Bovine 5	-	δ	δ	δ	α, δ
Bovine 6	-	NC	NC	NC	α, δ
Bovine 7	-	δ	δ	δ	α, δ
Bovine 8	-	δ	δ	δ	α, δ
Bovine 9	-	δ	δ	δ	α, δ
Bovine 10	-	NC	NC	NC	α, δ
Bovine 11	-	δ	δ	δ	α, δ
Bovine 12	+	δ	δ	DH	α, β, δ
Bovine 13	-	NC	NC	NC	α, δ
Bovine 14	-	NC	NC	NC	α, δ
Bovine 15	+	NC	α	β	α, β, δ
Bovine 16	-	δ	δ	δ	α, δ
Bovine 17	+	NC	δ	δ	α, δ
Bovine 18	+	δ	δ	δ	α, δ
Bovine 19	-	NC	NC	NC	α, δ
Bovine 20	-	NC	NC	NC	α, δ
Human 1	+	NC	α	DH	α, β, δ
Human 2	+	NC	α	DH	α, δ
Human 3	+	α	α	DH	α, β, δ
Human 4	+	NC	α	DH	α, β, δ
Human 5	-	δ	δ	δ	α, δ
Human 6	-	NC	α	α	α, δ
Human 7	-	NC	α	DH	α, δ
Human 8	-	NC	α	DH	α, δ
Human 9	-	NC	NC	NC	α, δ
Human 10	-	NC	α	α	α, δ
Human 11	-	δ	δ	δ	α, δ
Human 12	-	δ	δ	δ	α, δ
Human 13	+	NC	α	DH	α, δ
Human 14	+	NC	α	DH	α, β, δ
Human 15	-	δ	δ	δ	α, β, δ
Human 16	-	NC	α	α	α, β, δ
Human 17	-	NC	NC	NC	α, δ
Human 18	-	δ	δ	δ	α, δ
Human 19	+	NC	α	DH	α, β, δ
Human 20	+	NC	α	DH	α, β, δ

NC: Not change, and DH: Double hemolysis

Discussion

Staphylococci are the bacteria most frequently isolated from bovine mastitis and human infections. Diseases caused by this genus 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 staphylococci (da Silva *et al*., 2005). To date, the role of CNS as a cause of bovine mastitis and human infections and their hemolysin factors is not completely clear. So, in this study we investigated distribution of four hemolysins in bovine and human CNS and CPS isolates, phenotypically and genotypically. In this study, in addition to CPS samples (5 and 8 bovine and human isolates), 15 and 12 CNS samples isolated from bovine and human infections were used. We detected relatively large differences in the prevalence of hemolysins in human and bovine *Staphylococcus* isolates. All of the isolated CPS demonstrated hemolytic activity either alone or in combined forms while 25% of CNS strains were non hemolytic. A similar study in mastitic 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). Similar results were described by Watts and Owens (1987) who observed hemolysin production by *S. xylosus* and *S. sciuri*. Phenotypically, most of the bovine isolates showed delta hemolysis on sheep, horse and rabbit blood agar while human isolates produced alpha hemolysis which, in accordance with many studies has shown differences between the hemolysins produced by *S. aureus* isolated from bovine mastitis and from human infections (Silva and Cardoso, 2000; Larsen *et al*., 2002). Todar in 2005 suggested that most of the *S. aureus* isolated from human usually have beta hemolytic character, because the human platelets and monocytes are more sensitive to the alpha toxin and the majority of human isolates of *S. aureus* do not express ß-toxin. A lysogenic bacteriophage is known to encode the toxin. CPS isolated from animal mostly produce characterized beta toxin because of the sensitivity of animal erythrocytes to this toxin, but in this study the majority of CNS bovine isolates produced delta hemolysin which could be due to differences in hemotoxin production in CNS and CPS bovine strains. Also, in this study the double hemolysin observed in human isolates was higher than in bovine isolates, significantly ($P \leq 0.05$). This could possibly indicate that the human strains are more pathogenic, however, further investigations are necessary to validate this hypothesis. As shown in Table 2, all of the 49 CNS, CPS and reference strains tested by PCR were positive for alpha and delta hemolysin genes. These occurrence rates are in accordance with other authors (Silva and Cardoso, 2000; Larsen *et al*., 2002; Ebrahimi *et al*., 2009). Also, in combination with alpha and delta, beta hemolysin was observed in 40% of human strains which is significantly higher than bovine isolates ($P \leq 0.05$), that also supports the hypothesis that human strains have more pathogenicity. In the present study all isolates lacked gamma hemolysin expression. The genotypes of hemolysin of CNS and CPS in this study seemed to be not having correlation with the expression of their phenotypes. It might be influenced by many factors on the level of genetic or phenotypic.

The existence of *hla* and *hld* genes in *S. aureus* and CNS are important for these isolates related to staphylococcal infection cases that caused animal and human disease (Ariyanti *et al*., 2011). This study demonstrated that the *hla* and *hld* genes are widely distributed among *S. aureus* and CNS isolated from bovine and human. Also, the results from this study suggest that, in accordance with what is generally believed, some differences are apparent in hemolysin types among *Staphylococcus* strains of bovine and human origin. Furthermore, this study showed that CNS produces hemotoxins, and it might be important as an emerging pathogen.

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