

## Study on some characteristics of *Staphylococci* isolated from sheep sub clinical mastitis milk in Shahrekord, Iran

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### Abstract

**Introduction:** Staphylococci release a large number of enzymes. Some of these, such as coagulase, beta lactamase, hemolysins and biofilms are considered indices of pathogenicity. The aim of the current study was based on the isolation and identification of *Staphylococcus aureus* and coagulase negative Staphylococci (CNS) strains from sheep sub clinical mastitis and examining their biofilm, beta lactamase, hemolysins production and antibiotic resistance pattern.

**Materials and methods:** 55 Staphylococci strains were isolated from seventy cases of sheep subclinical mastitis. Thirty three were determined as *Staphylococcus aureus* (60%) and 22 (40%) as CNS. The hemolytic activity was evaluated by plating Staphylococci strains on 5% bovine blood agar. The biofilm assay was performed by using micro titer plates. Beta Lactamase production was detected by test tube iodometric technique and susceptibility to antimicrobial agents was determined for isolated strains by the disk diffusion method.

**Results:** Twenty six (78.8%) *S. aureus* strains were biofilm producers. For CNS (59.9%) strains were positive in biofilm production. Two isolates (6.06%), of *S. aureus* were  $\alpha$ , the same number  $\beta$  and 6 (18.2%) isolates were  $\delta$  hemolysin producers. Six isolates of CNS (27.27%) were  $\alpha$  and ten (45.45%)  $\delta$  hemolysin producers. Sixteen *S. aureus* (48.5%) and five CNS (22.72%) isolates were positive in beta lactamase production. The isolated Staphylococci show a low sensitivity pattern to methicillin and streptomycin.

**Discussion and conclusion:** A high percentage of strains make  $\alpha$  toxin that play a role in *S. aureus* biofilm formation. Twenty one out of 33 (63.63%) isolated Staphylococci were biofilm producers that can have deleterious effects because biofilm formation is thought to play an important role in the survival of virulent strains of Staphylococci. Sixteen out of 33 (48.5%) isolated *S. aureus* were positive in beta lactamase test, Excluding resistant to methicillin, all of these isolates show a marked sensitivities to other examined beta lactam drugs. High percentage of hemolysins, biofilm and beta lactamase production by isolated Staphylococci, suggest an important role of these virulence factors in the pathogenesis of isolated Staphylococci from mastitis sheep milk samples.

**Key words:** Mastitis, Sheep, Staphylococci, Biofilm, Beta lactamase, Hemolysin

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## Introduction

Sheep intra mammary infection (IMI) in its sub clinical form is the single most important factor affecting milk quality and quantity (1).

Several pathogens can cause mastitis but *Staphylococcus* spp. is the most frequently diagnosed causal microorganism of intra mammary infection (IMI) in sheep (2). Some species of this genus cause a variety of diseases by production of a series of enzymes and toxins, invasion of host cells and tissues.

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 (3). Additionally, beta-hemolysin is a sphingomyelinase that is highly active against sheep and bovine erythrocytes (4), while delta -hemolysin as well as alpha-hemolysin induce pore formation, perturbing the cell membrane permeability (5).

The biofilms consisted of micro colonies encased in extracellular polysaccharide material.

Bacteria inside biofilms have increased resistance to antimicrobial agents. The production of biofilms was correlated with pathogenicity and virulence of bacteria (6).

Many common bacterial pathogens exist in animals as biofilms. Mastitis is a typical animal disease where bacterial biofilms are believed to be involved based on histopathologic and ultra structural appearance of the bacteria within tissue (7).

The public health significance of *Staphylococci* isolated from milk and dairy products is important because these products can be a source of toxins and antibiotic-resistant strains for humans (8).

To treat mastitis successfully, means knowing the prevalence and the antimicrobial susceptibility patterns of

microorganisms isolated from the mammary gland.

The present study was undertaken in order to determine some phenotypic characteristics of *Staphylococci* isolates from sheep sub clinical mastitis in Shahrekord district in south west of Iran.

## Materials and methods

### Sample collection

The study covered 16 months from March 2011 to June 2012. A preliminary analysis for presence of sub clinical mastitis was done in 600 randomly selected native lactating sheep from 16 free ranging flocks in Shahrekord district, south west of Iran, via California mastitis test (CMT) (9).

CMT positive milk samples were aseptically obtained in sterile 10 ml tubes. The ice box containing milk samples were sent to microbiology lab of Veterinary College of Shahrekord university for the isolation of *Staphylococcus* species. In laboratory aliquots of 0.01 ml (10) of milk were streaked on blood agar plates containing 5% defibrinated sheep blood, (BA), (Merck, Darmstadt, Germany). The incubation was done aerobically at 37 °C for 24-48 h. The presence of more than 3 colonies of a similar morphotype was accepted as positive bacteriological finding (10).

### Identification of *Staphylococcus* species

One colony from similar morphotype colonies growing on above BA was subcultured on freshly prepared plates of mannitol salt agar (MSA), (Merck, Darmstadt, Germany) and incubated again aerobically at 37 °C for 24-48 h. Primary characterization of isolates was based on the gram stain, morphological and cultural characteristics. Colonies were tested with slide coagulase (using rabbit plasma) test (11). The catalase and oxidase tests were followed by examining the susceptibility profile of isolates towards bacitracin according to Quinn *et al.* (11).

The isolates were kept frozen at  $-70^{\circ}\text{C}$  in Tryptic soy broth containing 15% (v/v) glycerol, until the further examinations were carried out.

#### Assay for hemolytic activity

The hemolytic activity was evaluated by plating *Staphylococci* strains on 5% bovine blood for alpha and beta hemolysin production. The criteria for hemolysin identification were: complete lytic zone (transparent) with blurred edges for alpha-hemolysin and incomplete (non-transparent) lytic zone, which became complete with sharp edges after overnight incubation at  $4^{\circ}\text{C}$ , for beta-hemolysin (12). Delta hemolysin was determined by using the synergistic hemolysis method described by Hébert and Hancock (13), Fig. 1.

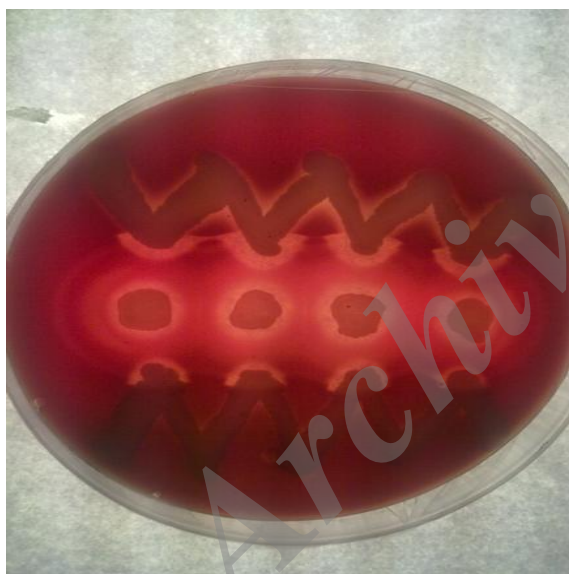


Fig. 1- Delta hemolysin determined by using the synergistic hemolysis of beta-hemolytic *S. aureus* seeded vertically (center) and the samples to be tested perpendicularly. Positive strains showing an increase in their hemolysis area at the extremity close to the beta-hemolytic *S. aureus*. Photo is taken from our experiments.

#### Antimicrobial Susceptibility testing

For antimicrobial susceptibility testing, isolates were incubated in Tryptic soy broth at  $37^{\circ}\text{C}$  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 (MH) agar, (Merck, Darmstadt, Germany), following the Clinical and Laboratory Standards Institute (CLSI), (14). The selected antibiotics for antibiogram were amoxicillin, kanamycine, penicillin, ciprofloxacin, tetracycline, gentamicin, methicillin, erythromycin, streptomycin and oxaciline.

Isolates were categorized as susceptible and resistant based upon interpretive criteria developed by the CLSI (14).

#### Biofilm and beta lactamase assays

Beta lactamase production was detected by test tube iodometric technique as described in reports (15).

The biofilm assay was performed by using micro titer plates as described by Tendolkar et al (16). Interpretation of biofilm production was according to the criteria's described by Stepanovic *et al.* (17). Based on these criteria's OD<sub>c</sub> (optical density cut-off value) is defined as: average OD of negative control +  $3 \times \text{SD}$  (standard deviation) of negative control, and the biofilms producers are categorized as: negative  $\leq \text{OD}_c$ , weak  $\text{OD}_c < \sim \leq 2 \times \text{OD}_c$ , moderate  $2 \times \text{OD}_c < \sim \leq 4 \times \text{OD}_c$  and strong biofilm producer  $> 4 \times \text{OD}_c$ . While " $\sim$ " stands for average of sample ODs.

#### Results

From 600 examined lactating sheep seventy (11.66%) were CMT positives which followed by bacteriological examinations. Cultures from fifty five CMT positive samples (78.6%) lead to isolation and identification of *Staphylococci*. Out of these 55 isolates, 33 (60%) were *S. aureus* and 22 (40%) were coagulase negative *Staphylococci*, (CNS). Details for hemolysins, biofilm and beta lactamase production by isolated *Staphylococci* is appeared in table 1.

Table 1: Hemolysin, biofilm and  $\beta$ -lactamase production by isolated Staphylococci from sub clinical mastitis sheep

Hemolysin	$\alpha$ no. (%)	$\beta$ no. (%)	$\delta$ no. (%)	$\alpha\beta$ no. (%)
<i>S. aureus</i>	2(6.06)	2(6.06)	6(18.18)	0(0)
CNS*	6(27.2)	0 (0)	10(45.4)	0 (0)
Biofilm	Strong no. (%)	Medium no (%)	Weak no (%)	Total no. (%)
<i>S. aureus</i>	0(0)	6(18.1)	20(60.6)	26(78.8)
CNS	0(0)	1(4.5)	12(54.5)	13(59.09)
$\beta$ -lactamase	Strong no. (%)	Medium no. (%)	Weak no. (%)	Total no. (%)
<i>S. aureus</i>	8(24.2)	6(18.1)	2(6.06)	16(48.4)
CNS	2(9.09)	2 (9.09)	1(4.5)	5(22.7)

\*Coagulase negative Staphylococci

In total 8 Staphylococci isolates were  $\alpha$  hemolysin positives out of them six (75%) were biofilm producers simultaneously.

Totally thirty nine out of 55 (70.9%) isolated Staphylococci were biofilm producers, out of them 16 isolates (41%) were positive in beta lactamase test.

Out of 33 isolates of *S. aureus* 16 (48.4) MRSA and 5 (15.1%) MSSA were recorded. The numbers for 22 isolates of CNS were 0 and 10 (45.4%) respectively.

Table 2 shows the result of overall antimicrobial susceptibility patterns irrespective of  $\beta$ -lactamase production.

Table 2: Antibiotic susceptibility responses of isolated Staphylococci from sub clinical mastitis sheep irrespective of  $\beta$ -lactamase production.

Staphylococci	<i>S. aureus</i> CNS			
	S no (%)	NS no (%)	S no (%)	NS no (%)
Amoxicillin	32 (96.96)	0 (0.0)	22 (100)	0 (0.0)
Kanamycine	26 (78.8)	0 (0.0)	17 (77.2)	0 (0.0)
Penicillin	27 (81.8)	0 (0.0)	18 (81.8)	0 (0.0)
Ciprofloxacin	33 (100)	0 (0.0)	22 (100)	0 (0.0)
Tetracycline	25 (75.5)	4 (12.1)	20 (90.9)	0 (0.0)
Gentamicin	28 (84.8)	0 (0.0)	20 (90.9)	0 (0.0)
Methicillin	5 (15.1)	16 (48.4)	10 (45.4)	0 (0.0)
Erythromycin	11 (33.3)	20 (60.6)	18 (81.8)	2 (9.1)
Streptomycin	16 (48.4)	2 (6)	7 (31.8)	2 (9.1)
Oxacillin	26 (78.7)	7 (21.2)	18 (81.8)	2 (9.1)
Ofloxacin	29 (87.8)	0 (0.0)	21 (95.4)	0 (0.0)

S= sensitive NS = non sensitive

The *S. aureus* isolates show a low sensitivity pattern to methicillin, erythromycin and streptomycin, while for CNS isolates only methicillin and streptomycin show this pattern.

## Discussion and conclusion

Sub clinical mastitis in small ruminant average from 5 to 30% (2), this rate covers our result of 11.66%, but is higher than our previous report of 4.75% (18).

The prevalence of bacterial isolates from clinically normal small ruminant's milk is affected by factors such as different hygiene and management practices followed on each farm, age and parity of the animal and type of milking (19,20).

In total twenty one out of 33 (63.63%) isolated Staphylococci were biofilm producers. This phenomenon can have deleterious effects because biofilm formation is thought to play an important role in the survival of virulent strains of Staphylococci. Moreover, biofilm formation has been shown to be positively correlated with resistance to antimicrobial agents (21). We don't recommend methicillin, erythromycin and streptomycin for treating ewe's mastitis cases because of low sensitivity pattern of recovered agents to these antimicrobials.

Eight out of 33 (24.248%), of our *S. aureus* isolates were  $\alpha$ , hemolysin producers, out of them, 6 (75%) were biofilm producers simultaneously. Nicky and Toole (22) showed a role for  $\alpha$  hemolysin in *S. aureus* biofilm formation and that this toxin appears to be required for cell-to-cell interactions. A high percentage of strains make this toxin, and it is toxic to a wide range of mammalian cells, but environmental factors appear to play a role in alpha-hemolysin expression (3).

Only 6% of *S. aureus* isolates were positive in  $\beta$  hemolysin production that is much lower from bovine mastitis isolates reported elsewhere (4). Regarding ovine isolates published data is scarce.

Six out of 33 (18.18%) of our *S. aureus* isolates were  $\delta$  hemolysin producers, This is much lower from reports that recorded 80%-97%  $\delta$  hemolysin production by *S. aureus* isolates (23). This toxin is capable of causing membrane damage in a variety of mammalian cells, as well as sub cellular structures such as membrane-bound organelles, spheroplasts, and protoplasts (24).

George et al, reported absent of delta-hemolysin expression in *S. aureus* isolates suggests that relative gene function is suppressed in these isolates (25).

Ten out of 22 (45.45%) isolates of CNS were  $\delta$  hemolysin producers. Reports estimated a 40%- 80% of CNS have the ability to produce this toxin (26), with a detergent action on the membranes of various cell types. It is indicated that different genes might be responsible for the production of this toxin in different CNS species (27).

Sixteen out of 33 (48.5%) isolated *S. aureus* were positive in beta lactamase test, excluding resistant to methicillin, all of these isolates show a marked sensitivities to other examined beta lactam drugs. The sensitivities may be due to high level of penicillin receptors (PBPs) or high accessibility of these receptors due to a lack of permeability barriers created by autolytic enzymes in the cell wall, which can result in killing bacteria and contribute to high sensitivity rates seen in  $\beta$ -lactamase producers.

In conclusion, the high percentage of hemolysins, biofilm and beta lactamase production by isolated *Staphylococci* obtained in this work; suggest an important role of these virulence factors in the pathogenesis of isolated *Staphylococci* from ewe's mastitis milk samples. The *S. aureus* isolates show a high sensitivity pattern to most examined antibiotics. Methicillin followed by erythromycin and streptomycin were found to be low active drugs against isolates from mastitis milk samples.

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## مطالعه برخی خصوصیات استافیلوکوکسی‌های جدا شده از شیر میش‌های مبتلا به ورم پستان تحت بالینی در شهر کرد، ایران

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### چکیده

**مقدمه:** استافیلوکوکسی‌ها آنزیم‌های فراوانی در محیط آزاد می‌کنند که برخی از آن‌ها نظیر کوآگولاز، بتا لاکتاماز، همولایزین‌ها و بیوفیلیم‌ها جزء اندیس‌های بیماری‌زایی این باکتری‌ها محسوب می‌شوند. هدف مطالعه حاضر، جداسازی و تشخیص استافیلوکوکوس اورئوس و استافیلوکوکوس‌های کوآگولاز منفی از گوسفندان مبتلا به ورم پستان تحت بالینی و آزمایش تولید بیوفیلیم، بتا لاکتاماز و همولایزین‌ها و نیز الگوی مقاومت آنتی‌بیوتیکی آن‌ها می‌باشد.

**مواد و روش‌ها:** در این مطالعه، ۵۵ جدایه استافیلوکوکسی به دست آمد که ۳۳ مورد آن (۶۰ درصد) استافیلوکوکوس اورئوس و ۲۲ مورد (۴۰ درصد) استافیلوکوکسی کوآگولاز منفی تشخیص داده شد. فعالیت همولایتیک جدایه‌ها با کشت آن‌ها بر سطح محیط ژلوز خون‌دار (تهیه شده با خون گاو) ارزیابی شد. تولید بیوفیلیم با روش پلِت میکروتیتر ارزیابی و تولید بتا لاکتاماز با روش یودومتریک و حساسیت آنتی‌بیوتیکی با روش دیسک دیفیوژن تعیین شد.

**نتایج:** ۲۶ مورد (۷۸/۸) از جدایه‌های استافیلوکوکوس اورئوس و ۵۹/۹ درصد از استافیلوکوکسی‌های کوآگولاز منفی قادر به تولید بیوفیلیم بودند. از جدایه‌های استافیلوکوکوس اورئوس دو مورد همولیز آلفا (۶/۰۶ درصد)، دو مورد همولیز بتا و ۶ مورد (۱۸/۲ درصد) همولیز دلتا تولید کردند. از استافیلوکوکسی‌های کوآگولاز منفی ۶ جدایه (۲۷/۲۷) همولیز آلفا و ۱۰ جدایه (۴۵/۴۵ درصد) همولیز دلتا تولید کردند. در جدایه‌های استافیلوکوکوس اورئوس ۲۶ مورد (۷۸/۸ درصد) و استافیلوکوکسی‌های کوآگولاز منفی پنج مورد (۲۲/۷۲ درصد) تولید آنزیم بتا لاکتاماز کردند. استافیلوکوکسی‌های جدا شده حساسیت پایینی نسبت به متی‌سیلین و استرپتومایسین نشان دادند.

**بحث و نتیجه‌گیری:** درصد بالایی از جدایه‌ها همولایزین آلفا تولید کردند که نقش مهمی در تولید بیوفیلیم توسط استافیلوکوکوس اورئوس ایفا می‌نماید. ۲۱ جدایه از ۳۳ جدایه (۶۳/۶۳ درصد) استافیلوکوکوس‌ها قادر به تولید بیوفیلیم بودند که می‌تواند اثرات بدی داشته باشد. چون به نظر می‌رسد تولید بیوفیلیم نقش مهمی در ابقا این باکتری داشته باشد. ۱۶ جدایه از ۳۳ جدایه (۴۸/۵ درصد) استافیلوکوکوس اورئوس در آزمایش تولید بتا لاکتاماز مثبت شدند. صرف نظر مقاومت به متی‌سیلین تمامی این جدایه‌ها حساسیت قابل توجهی به آنتی‌بیوتیک‌های بتا لاکتاماز مثبت بودند. آزمایش نشان دادند. درصد بالای تولید همولیزین، بیوفیلیم و بتا لاکتاماز بیانگر نقش مهم این عوامل حدت در بیماری‌زایی استافیلوکوکسی‌های جدا شده از نمونه شیرهای ورم پستان میش است.

**واژه‌های کلیدی:** ورم پستان، گوسفند، استافیلوکوکسی، بیوفیلیم، بتا لاکتاماز، همولیزین

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تاریخ دریافت: ۱۳۹۲/۰۶/۱۲ - تاریخ پذیرش: ۱۳۹۲/۱۲/۱۰