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Research Paper

PHENOTYPIC AND MOLECULAR CHARACTERIZATION HEMOLYSINS OF STAPHYLOCOCCUS AUREUS ISOLATED FROM MASTITIC COW'S MILK IN EGYPT

Gamal Younis[1], Amal Awad[1*] and Basma Shabana[1]

*Corresponding Author: Amal Awad, ✉ amalabdo@mans.edu.eg

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One important feature of *Staphylococcus aureus* virulence is due to its ability to attack the cell membrane of host cell with a large array of membrane-damaging toxins and peptides such as hemolysins. Therefore, this study was undertaken to perform phenotypic and genotypic characterization of *S.aureus* hemolysins isolated from milk of cows with clinical mastitis. A total of 173 milk samples were collected from two dairy farms and were subjected for *S.aureus* bacteriological isolation procedures, 55 *S.aureus* isolates have been recovered with a percentage of 31.79%. *S.aureus* isolates were evaluated for their Alpha (α) and Beta (β) hemolysins production on sheep and bovine blood agar. Alpha hemolysin were detected in 50 (90.90%) strains, beta-hemolysin were detected in 47 (85.45%) strains either alone or in combined. However, non-hemolytic *S.aureus* strains were detected in 5 (9.09%) strains. On titration, α-toxin titers were much more than that for β-toxin. Based on PCR results, the overall prevalence rate of hla was higher than hlb and these results were correlated with their phenotypic hemolytic activity on blood agar plates. In conclusion, the high prevalence rate of α and β-hemolysins in *S.aureus* isolated from mastitis suggests that hemolysins could have significant role in pathogenesis of mastitis, in addition, the capability of *S.aureus* strains to produce alpha and beta hemolysin indicates that these toxins might be necessary for the establishment of the *S.aureus* in mammary glands.

Keywords: S.aureus, Mastitis, Hemolysins, Phenotypic, Genotypic

INTRODUCTION

Mastitis considered one of the most common worldwide disease of cattle which causing great economic losses. *S.aureus* is one of the most frequently causative agent of bovine clinical mastitis (Sawant *et al.*, 2009;

[1] Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Mansoura University, Mansoura, 35516, Egypt.

Simojoki *et al.*, 2012; and Kot *et al.*, 2012a and 2012b).

S.aureus produces a wide array of virulence elements including, enzymes and toxins, which are responsible for the invasion of host cells such as hemolysins (da Silva *et al.*, 2005). Hemolysins are considered the most important virulent elements in development of the disease (Dinges *et al.*, 2000; and Ariyanti *et al.*, 2011). Typing and titration of hemolysins is a pointer of pathogenicity due to its hemolytic, dermonecrotic and neurotoxic effects (Dinges *et al.*, 2000).

Hemolysis of staphylococci are classified into 4 different types including alpha (α), beta (β), gamma (γ) and delta (δ) (Aarestrup *et al.*, 1999). Alpha (α) toxin is a heptamer pore-forming exotoxin that lyses erythrocytes of rabbit as well as it is toxic to human epithelial cells (Gouaux *et al.*, 1994). Beta-hemolysin (β) is a sphingomyelinase, it also has a high activity against erythrocytes of sheep and bovine (Larsen *et al.*, 2002). Beta toxin known as the hot-cold toxin as it has a unique activity on sheep blood agar; it interacts with sheep red blood cells but does not lyse them at 37 °C. It then lyses the red cells if they are placed at 4 °C (Huseby *et al.*, 2007).

The overall aims of this study were to determine the hemolytic activity of *S.aureus* isolated from mastitic cow's milk on sheep and bovine blood agar and to characterize *S.aureus* hemolysins genes using PCR assay.

MATERIAL AND METHODS

Sampling

A total of 173 milk samples were collected from two cows dairy farms located at Dakahalia and Damietta provinces in the period between July and October 2016. Milk samples were collected from mastitic cows showing clinical signs of inflammation after physical inspection of udder, 10 ml of milk samples were collected from the infected quarter under complete aseptic condition in sterile Falcons tubes after cleaning the udder, disinfection of teats and discard the first streams of milk, milk samples were then transported in ice box directly to the laboratory for bacteriological examination procedures. All milk samples were kept at 4 ^{o}C until examination with a minimal of delay.

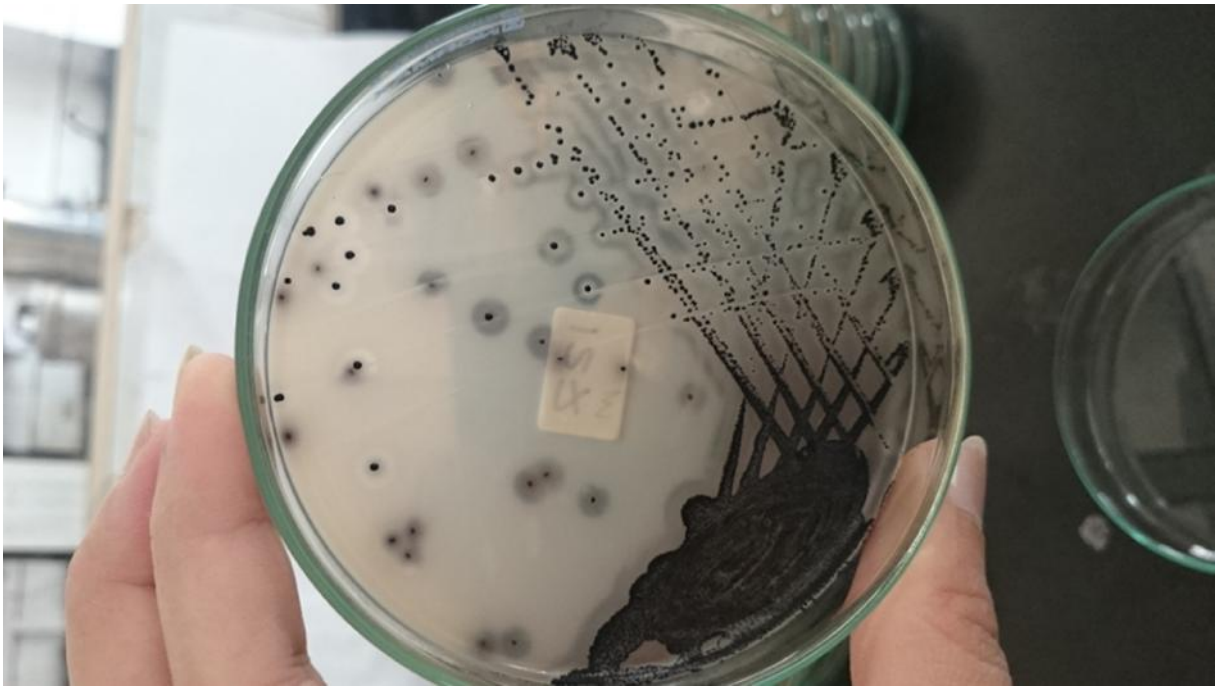
Bacteriological Examination

Isolation of *S.aureus* was done following the protocol previously recorded by Wang *et al.* (2012). In brief, aliquots of 3 ml of milk samples were inoculated into 27 ml of tryptone soya broth (TSB; Oxoid, UK) with 7.5% NaCl and incubated at 37 °C for 18-24 h. A loopful from the inoculated broth was streaked onto Baird-Parker agar plates (Oxoid, UK) containing 5% egg yolk and1% potassium tellurite and incubated at 37 °C for 24 h. Presumptive *S.aureus* colonies (black colored colonies surrounded by halos) (Figure 1) were purified by sub-culturing on tryptone soya agar plates (TSA; Oxoid, UK) plates. Presumptive *S. aureus* colonies were gram stained and subjected for coagulase test and other biochemical tests (Kumar *et al.*, 2010). Further identification of *S.auerus* was done by using API Staph system (bioMérieux, Marcy l'Etoile-France).

Haemolytic Activity

The phenotypic hemolytic activity for *S.aureus* isolates was estimated by using blood agar base (Oxoid) supplemented with 5% sheep and bovine blood for estimation of alpha and beta-hemolysin respectively (Quinn *et al.*, 1994). *S.aureus* Strains

Figure 1: Colony Characters of S.aureus on Baird-Parker Agar



were streaked onto the surface of blood agar plates and incubated for 24 h at 37 °C and also for 48 h. The identification criteria for hemolysin were: complete zone of hemolysis with unclear edges for α-hemolysin (Figure 2). For beta-hemolysin, incomplete zone of hemolysis, which with incubation for overnight at 4 °C became complete with sharp edges (Figure 3) (Quinn *et al.*, 1994; and da Silva *et al.,* 2005)

Titration of Hemolysins

For the tube titration, it was done according to (Haque and Baldwin, 1964) in brief, *S.aureus* colonies that showed hemolysis were incubated in tubes containing trypton soya broth in an atmosphere containing 20% carbon dioxide at 37 °C for 48 hr, and the supernatant fluids were harvested by centrifugation, the supernatant fluids were titrated against 1% suspensions of sheep and human red blood cells. The tubes were incubated for 1 hr at 37 °C, and were then refrigerated overnight. The titer of the supernatant fluids was recorded after incubation at 37 °C and after overnight refrigeration (Figure 4).

Figure 2: ~-Haemolysis of S.aureus on Human Blood Agar Plates

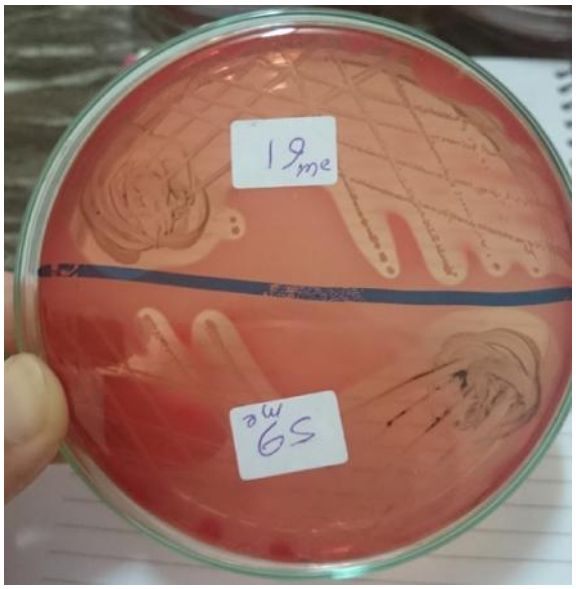
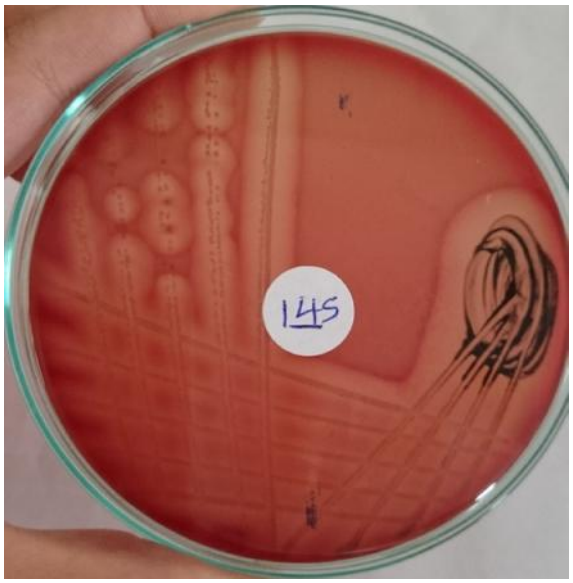


Figure 3: -Haemolysis of S.aureus on Sheep Blood Agar



Genotypic Characterization of Hemolysins

DNA extraction was performed using PureLink Genomic DNA extraction kit (Invitrogen, Carlsbad, CA) following the manufacturer guidelines. PCR assay was carried out on *S.aureus* isolates to detect *hla* and *hlb* genes as previously reported by Fei *et al.* (2011) the oligonucleotide primers sequences and their amplicons size are listed in Table 1. The PCR reaction for each gene was performed in a total volume of 25 µl consists of 12.5 µl master mix (Emerald Amp GT PCR-(Takara) Code No. RR310A, 1 µl from each primer (Metabion-Germany), 6 µl of the DNA template and 4.5 µl nuclease free water. The amplification program included one denaturation cycle at 94 °C for 5 min, then secondary denaturation at 94 °C for 30 sec, annealing at 60 °C for 45 sec, extension 72 °C for 45 sec for 35 cycles and final extension at 72 °C for 10 min. The amplified product for each gene was separated by 1.2% agarose gel stained with ethidium bromide and visualized by UV transillunination and photographed.

Figure 4: -Haemolysin Titre with Washed Sheep RBCs Showed 50% Haemolysis at the Dilution 1\32

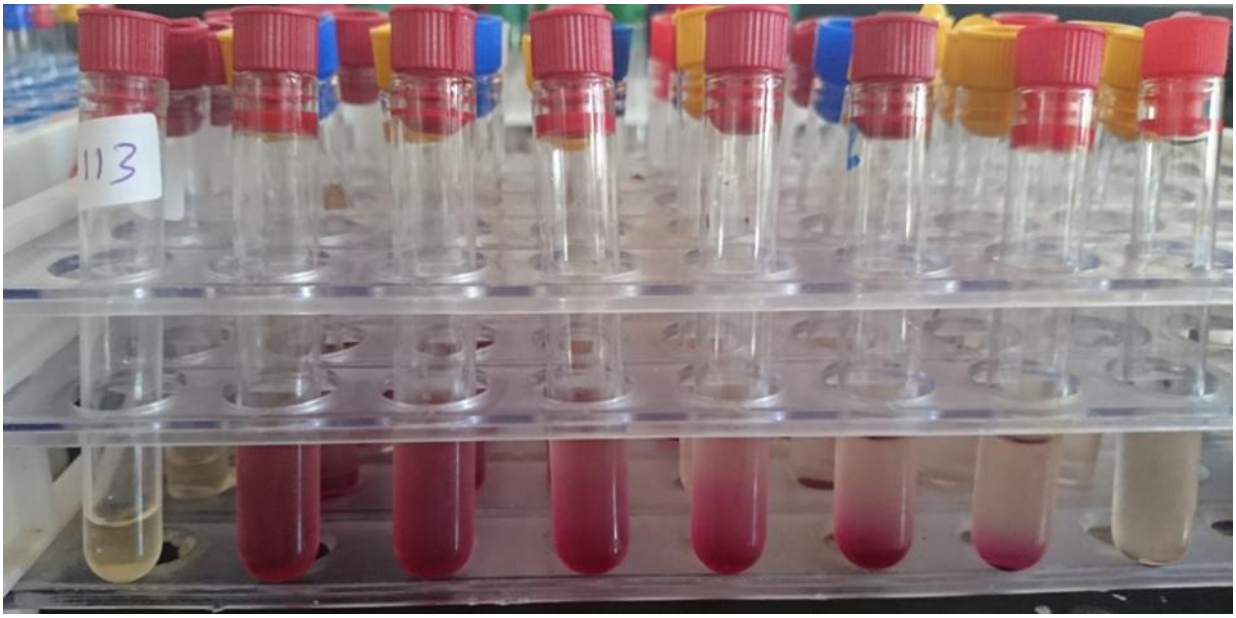


Table 1: Oligonucleotide Primers Sequences

Gene	Primer Sequence (5'-3')	Length of Amplified Product	Reference
hla	GAAGTCTGGTGAAAACCCTGA	704 bp	Fei *et al* . (2011)
	TGAATCCTGTCGCTAATGCC		
hlb	CAATAGTGCCAAAGCCGAAT	496 bp	
	TCCAGCACCACAACGAGAAT		

RESULTS AND DISCUSSION

Staphylococci are considered the most important etiological agents associated with bovine mastitis (Sawant *et al.*, 2009; Simojoki *et al.*, 2012; and Kot *et al.*, 2012a and 2012b). In the current study, 173 milk samples collected from clinical cases of mastitis were investigated for the presence of *S.aureus* of which 55 (31.79%) isolates were identified as *S.aureus* positive. Interestingly, all isolated *S.aureus* strains were tested positive for coagulase test. Coagulase production by *S.aureus* has been related to pathogenicity of this organism and has also been used as an important criterion for the identification of the organism phenotypically (Radostits *et al.*, 2000; Kateete *et al.*, 2010; Bhati *et al.*, 2016; Al-Ashmawy *et al.*, 2016; and Awad *et al.*, 2017).

S.aereus pathogenicity is contributed to the secretion of several virulence factors responsible for the development of infection (Taponen and Pyorala 2009; and Soares *et al.*, 2011). There is diversity in the virulence factors produced by *S.aureus* strains isolated from bovine mastitis (Melchior *et al.*, 2009; and Capurro *et al.*, 2010). Hemolysins are considered the most important cytolytic exotoxins produced by *S.aureus* which affecting the cell membrane. Alpha (α)-hemolysin is the main pathogenicity element because it has hemolytic, neurotoxic and dermonecrotic action (Berube and Wardenburg, 2013; Olivia and Subtil 2014; and Yadav *et al.*, 2015). Beta (β)-hemolysin is aspingomyelinase and it was expressed by most strains isolated from bovine suffering from intra-mammary infections (Cifrian *et al.*, 1996; and Larsen *et al.*, 2002).

In this study, the hemolytic activity produced by *S.aureus* was detected by measuring its phenotypic expression on sheep and bovine blood agar, 90.90% of *S.aureus* isolates produced α-hemolysin with different degrees of hemolysis either week (12.73%), moderate (41.82%), or high (36.36%). For β-hemolysin, 85.45% of *S.aureus* strains were considered β-hemolysin producer with different degrees either weak (29.09%), moderate (14.55%) or high (41.82%) either alone or in combined forms (Table 2). Similarly, Al-Ashmawy *et al.* (2016) detected ~-hemolysins in all isolated *S.aureus* from milk and dairy products and Kot *et al.* (2013) recorded that 93.9% of *S.aureus* strains from cows with mastitis showed hemolytic activity, among them 72.7% and 21.2% produced α- and β-hemolysin, respectively. In addition, Da Silva *et al.* (2005) found that 76.7%

Table 2: Hemolysis Degrees of the Isolated S.aureus Strains

Degree of Haemolysis	*α-haemolysis*		*β-haemolysis*	
	No. of Isolates	Percentage	No. of Isolates	Percentage
Negative	5	9.09%	8	14.55%
Weak	7	12.73%	16	29.09%
Moderate	23	41.82%	8	14.55%
High	20	36.36%	23	41.82%
Total	55	100%	55	100%

of *S.aureus* isolates were producers of alpha-hemolysin, either alone or combined. In another study conducted by Yadav *et al.* (2015), 100% of *S.aureus* isolates produce alpha-toxin whereas beta-toxin was produced by 68.75%, Upadhyay and Kataria (2010) also reported production of alpha toxin by all the isolates studied in his study from bovine and goat mastitic milk. Kenny *et at.* (1992) detected 94.3% of *S.aureus* from bovine mammary glands produced alpha-hemolysin

In the present study, 5 (9.09%) strains out of 55 *S.aureus* strains were non hemolytic which is agreed with Yadav *et al.* (2015) who recorded 9.37% of the studied *S.aureus* isolates were non hemolytic. Similarly, Graber *et al.* (2013) recorded 0-2% of non-hemolytic S.*aureus* in their study. Sanjiv and Kataria (2007) and Upadhyay and Kataria (2010) could not detect a hemolytic *S.aureus* isolates in their work, Salasia *et al.* (2004) also reported 10 non-haemolytic isolates out of 35 *S.aureus* isolates from bovine subclinical mastitis.

In the present study, 50 isolates produced alpha-toxin among them, 17 (30.91%) isolates produced titre of 1:256. For beta-toxin, 47 strains were beta toxin producer of which 16 (29.09%) strains produce a titre of 1:64. The titres of beta-toxin were also much less than that for alpha-toxin (Table 3). These results were in complete agreement with those of Sanjiv and Kataria (2007) and Upadhyay and Kataria (2010) who reported production of alpha-haemolysin by all the isolates with high alpha-toxin and the titre of alpha toxin

Table 3: Hemolysins Titres of the Isolated S.aureus Strains

Haemolysin End Titre	*α-haemolysin*		*β-haemolysin*	
	No. of Samples	Percentage	No. of Samples	Percentage
Negative	2	3.64%	8	14.55%
4	3	5.45%	3	5.45%
8	1	1.82%	1	1.82%
16	7	12.73%	9	16.36%
32	9	16.36%	9	16.36%
64	9	16.36%	16	29.09%
128	7	12.73%	6	10.91%
256	17	30.91%	3	5.46%
Total	55	100%	55	100%

Figure 5: Agarose Gel Electrophoresis Showing Amplification of 704 bp Fragment Using hla Primer, L: 100 bp DNA Ladder, Lane 2-12: Positive Samples, Lane 1: Negative Sample, Pos: Positive Control, Neg: Negative Control

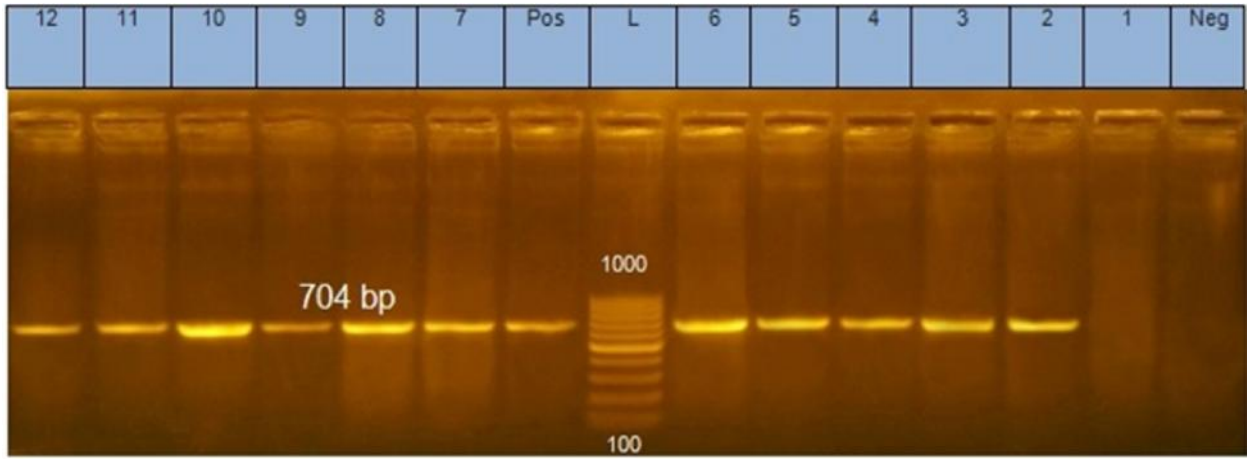
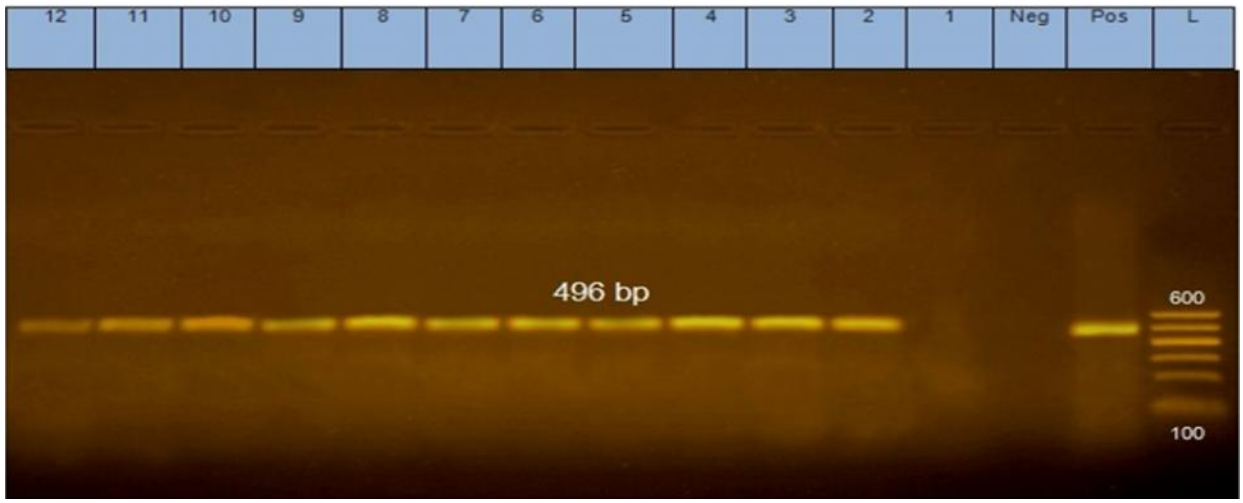


Figure 6: Agarose Gel Electrophoresis Showing Amplification of 496 bp Fragment Using hlb Primer, L: 100 bp DNA Ladder, Lane 2-12: Positive Samples Lane 1: Negative Sample, Neg: Negative Control, Pos: Positive Control



was much higher than that of beta toxin. These results confirmed that there is no difference in the qualitative and quantitative production of these toxins.

The overall prevalence of *hla* gene was 90.90% which was higher than that of *hlb* (85.45%) gene (Figures 5 and 6). These results were correlated with their hemolytic activity on blood agar plates. The same observations were recorded by Haveri *et al.* (2007) who recorded the prevalence of 97.4% and 76.7% for the *hla* and *hlb* genes, respectively. Salasia *et al.* (2011) also recorded the prevalence of 81.81% for *hla* gene harboring isolates. In addition, Yang *et al.* (2012) recorded the prevalence rate of 85% and 82% for *hla* and *hlb* gene respectively. In contrary, lower prevalence rates for both genes were recorded by many other authors (Booth *et al.,* 2001; Wang *et al.,* 2011; and Coelho *et al.,* 2011).

In conclusion, the results from the present study suggest that alpha and beta hemolysins may play a vital role in pathogenesis of bovine mastitis. The production of alpha and beta hemolysin by *S.aureus* may aid in the establishment of *S.aureus* in mammary glands tissue and also indicates that mammary glands are a significant reservoir of hemolytic staphylococci.

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Hyderabad, INDIA. Ph: +91-09441351700, 09059645577
E-mail: editorijasvm@gmail.com or editor@ijasvm.com
Website: www.ijasvm.com