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Detection of *hla* and *hlb* gene in *Staphylococcus aureus* obtained from open wounds in cattle

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Abstract
Staphylococcus aureus is an opportunistic skin pathogen of humans and animals with clinical manifestations including skin and soft-tissue infection, sepsis and pneumonia. Haemolysin is one of the important virulence factor of *S. aureus.* In the present study out of 63 swabs from wound samples with pus in cattle collected from areas in and around Bikaner city, 30 *S. aureus* isolate were isolated with a recovery of 47.60%. All 30 samples were genotypically confirmed by 23S rRNA based ribotyping method. All the 30 isolates were typable for haemolysin genes where *hla* gene was detected in 29 (96.7%) and *hlb* gene in 25(83.3%) isolates. On sheep blood agar 22 *S. aureus* isolates showed haemolysis of which 11 isolates (36.7%) produced complete haemolysis, six (20%) produced incomplete haemolysis and five (16.66%) produced both types of hemolysis. Haemolysis was not shown by eight isolates. Hence *S. aureus* isolates obtained from open wound in cattle showed higher percentage of haemolysin both genotypically and phenotypically.

Keywords: Cattle, Haemolysin, *hla* & *hlb* gene, Wound

Introduction
Staphylococcus aureus is an important commensal and pathogen that can be found on the skin, mucosa, and in the environment of a wide variety of animals, including humans. *S. aureus* is a non-motile, non-spore producing, facultative anaerobic firmicute bacterium belonging to the Staphylococcaceae family (Fluit, 2012) [16]. Many distinct cell types are affected by alpha haemolysin, including endothelial cells, blood cells, and platelets, epithelial cells (Berube and Wardenburg, 2013) [5]. Alpha haemolysin is a pore-forming protein that forms an oligomeric beta-barrel (Menestrina *et al.*, 2001) [23]. Pore creation on the membranes of susceptible host cells causes changes in ion gradients, membrane integrity loss, induction of stress-signaling pathways, and finally cell death (Bhakdi and Jensen, 1991; Husmann *et al*., 2006) [6, 18].
The goal of current study was to characterise *S. aureus* obtained from open wounds in cattle for haemolytic property on a phenotypic and genotypic level.

Materials and Methods
Sample collection
A total of 63 swabs from pus-filled wound samples from cattle belonging to various veterinary clinics in and around the city (Bikaner) were obtained. All samples were taken aseptically from skin wounds in cattle using sterile absorbent cotton swabs soaked in nutritional broth and promptly transported to the laboratory over ice for *S. aureus* isolation and identification using normal conventional procedures (Cowan and Steel, 1975; Quinn *et al*, 1994) [12, 26].

Genotypic confirmation of *Staphylococcus aureus*
We used Nachimuttu *et al*., (2001) [24] method for genomic DNA extraction of *S. aureus*, while Sambrook *et al*., (1989) [28] method for quantification. All 30 isolates from cow wound samples were genotyped using 23S rRNA based ribotyping in this study as per method described by Straub *et al*., (1999) [30]. The master mix was prepared by mixing GENETAQ Green Master Mix (2X) 12.5mL, primers (25 pM/µl) 0.5 µl, DNA template 3.0 µl and Nuclease free water to make 25.0 µl. Primer used for 23S rRNA ribotyping mentioned in table 1.

Table 1: Primers used for detection of genes in *S. aureus* isolates obtained from open wounds in cattle

S. No.	Target Gene	Primer sequence	Size (bp)	Reference
1	23S rRNA	F: 5´-ACGGAGTTACAAAGGACGAC-3´	1250	(Straub *et al.*, 1999)
		R: 5´-AGCTCAGCCTTAACGAGTAC-3´		
2	*hla*	F-5'-GGTTTAGCCTGGCCTTC-3'	534	(Booth *et al.*, 2001)
		R-5'-CATCACGAACTCGTTCG-3'		
3	*hlb*	F-5'-GCCAAAGCCGAATCTAAG-3'	833	(Booth *et al.*, 2001)
		R-5'-CGCATATACATCCCATGGC-3'		

Amplification of *hla* and *hlb* gene

For the amplification of the *hla* and *hlb* genes, Booth *et al.* (2001) [9] method was employed. Table-1 shows the sequences of two primers used for the amplification *hla* and *hlb* genes. The master mixture was made in the same way that 23S rRNA ribotyping was done. The amplification was done in thermocycler with initial denaturation at 94 ºC for 30 sec and followed by 30 cycles primer annealing 53 ºC for 1 min, primer extension 72 ºC for 30 sec. Final extension was done at 72 ºC for 7 min and reaction is terminated by holding at 4º C. The PCR products (for genus specific PCR) were subjected to electrophoresis at 4 V/cm for 45 minute in 1.5 per cent agarose gel prepared in 1 x TBE buffer containing 0.5 ng/ml of ethidium bromide. The PCR products (5µl) were run along with 100 b.p. DNA ladder (Invitrogen) and amplicons were visualized under UVP Gel Doc Bio-imaging System.

Results and Discussion

Genotypic confirmation of *Staphylococcus aureus*

In this study, all 30 isolates from cattle wound samples were examined to 23S rRNA based ribotyping for genotypic verification after being identified by standard microbiological procedures. All of the isolates had a 1250 bp amplicon, which confirmed that they were *S. aureus* (Fig.1). Other researchers from the same lab have employed a genotypic method based on 23S rRNA ribotyping for *S. aureus* identification in the past, including Upadhyay *et al.* (2010), Rathore *et al*. (2012), Khichar and Kataria (2014), Yadav *et al*. (2015), Bhati *et al*. (2018), and Singh *et al* (2018) [31, 27, 22, 34, 7, 29].

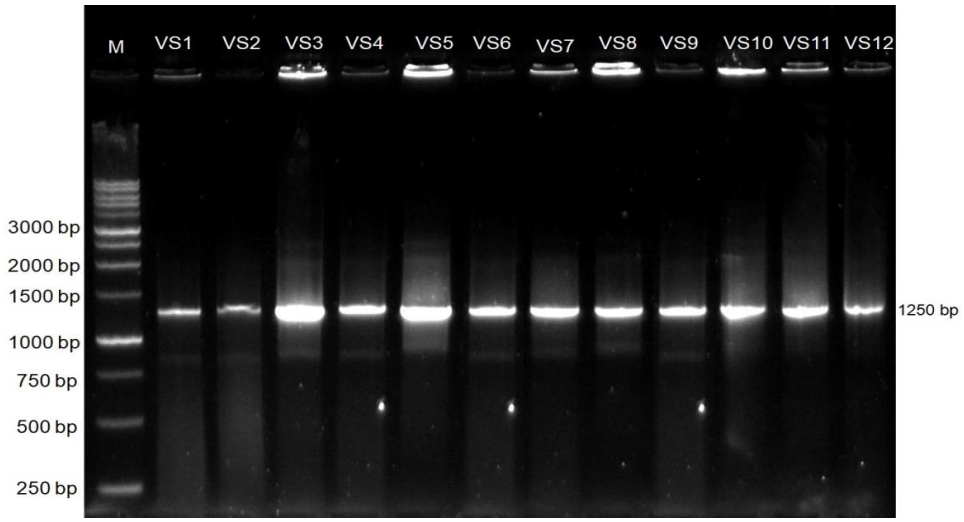

Fig 1: PCR amplicons of 23S rRNA ribotyping of *S. aureus* isolates

Haemolytic property: To analyse the hemolysis pattern, all 30 *S. aureus* isolates from cattle wound samples were aerobically cultured for 24 hours at 37 °C on sheep blood agar. Different forms of hemolysis were seen in the isolates (Table. 2). 11 isolates (36.7 percent) produced complete haemolysis, six isolates (20 percent) produced incomplete haemolysis, five isolates (16.66 percent) produced both types of hemolysis and eight isolates (26.66 percent) did not produce any haemolysis. As a result, 22 (73.3%) hemolytic and eight (26.7%) non-hemolytic *S. aureus* isolates were found in the current investigation.

Many workers have reported haemolysis on sheep blood agar by *S. aureus* isolates of various origins with variable results. Our finding matched those of Karmakar *et al*. (2016) [21] who reported 40% of *S. aureus* isolates that were able to produce a clearing zone surrounding their growth on blood agar media. Qureshi and Kataria (2012) [25] found that out of 40 camel skin *S. aureus* isolates 40% isolates produced complete haemolysis, 27.5% isolates produced incomplete haemolysis and 32.5% isolates produced both types of haemolysis but there was no ahaemolytic isolate.

Table 2: Haemolysis pattern of *S. aureus* isolated from wound samples of cattle

S. No.	Type of haemolysis	Isolate ID	No. of isolates
1.	Complete	VS3, VS5, VS8, VS9, VS16, VS17, VS23, VS24, VS26, VS27, VS28	11(36.66%)
2.	Incomplete	VS11, VS12, VS14, VS19, VS20, VS30	06(20%)
3.	Both	VS1, VS7, VS21, VS25, VS13	05 (16.66%)
4.	No hemolysis	VS2, VS4, VS6, VS10, VS15, VS18, VS22, VS29	08(26.66%)

In contrast to present study, a higher prevalence (86.66%) of beta haemolysis was reported by Anosike *et al*. (2019) [4] for *S. aureus* isolates from the wounds of hospital patients while two (13.33%) isolates exhibited alpha haemolysis on blood agar. Jahan *et al*. (2015) [19] reported 100% isolates showing β-hemolysis on 5% sheep blood agar, which is in contrast to present study. A double zone of both complete and incomplete haemolysis together was reported in five (16.66%) isolates in our study. Contrary to present results both types of haemolysis were reported by Wald *et al*. (2019) [32] in 61% field isolates and 37% isolates were showed β-hemolysis. Two percent of *S. aureus* isolate were ahaemolytic in nature.

In the present study eight (26.66%) isolates did not produce any haemolysis and were considered ahaemolytic. Many other workers have recorded a variable percentage of non-haemolytic *S. aureus* from different sources *viz*. 61% by Akineden *et al*. (2001) [2]; 57.13% by Coelho *et al*. (2009) [10] and 8.1% by El-Jakee *et al*. (2010) [14]. In contrast to present observations, Boerlin *et al.* (2003) [8] did not detect incomplete haemolysis on the blood agar plate by *S. aureus* isolates.

hla and *hlb* gene

The pathogenicity of *S. aureus* is also associated to production of these hemolysins as they contribute to disease causation in mammalian species (da Silva *et al*., 2005) [13]. The *hla* gene was present in five isolates showing single band of 534 bp (Fig.2) while one isolate carried *hlb* gene producing single band of 833 bp (Fig.3) and 24 (80%) isolates were typable for both the genes. Hence, *hla* gene was identified in total 29 (96.7%) and *hlb* gene in 25 (83.3%) isolates (Table 3).

Table 3: Detection of haemolysin genes (*hla* and *hlb*) in *S. aureus* isolates from wound sample of cattle

S. No.	Gene type	Isolate numbers	Total isolates	Amplicon size(bp)
1.	*hla*	VS3, VS4, VS8, VS9, VS11	5 (16.7%)	534
2.	*hlb*	VS19	1 (3.3%)	833
3.	Both *hla* and *hlb*	VS1, VS2, VS5, VS6, VS7, VS10, VS12, VS13, VS14, VS15, VS16, VS17, VS18, VS20, VS21, VS22, VS23, VS24, VS25, VS26, VS27, VS28, VS29, VS30	24 (80%)	534, 833

The prevalence of *hla* gene in the present study was in accordance with Wang *et al*. (2016) [33] who observed 94.3% prevalence of *hla* gene. Likewise, Yang *et al*. (2012) [35] recorded the prevalence of *hla* gene 85%, Acosta *et al*. (2018) [1] recorded *hla* gene in 88% *S. aureus* isolates, Haveri *et al*. (2007) [17] recorded 97.4% *hla* gene prevalence and Yu *et al*. (2015) [36] detected *hla* gene in 95.3% isolates of 128 *S. aureus* from human patients with skin and soft tissue infections. However, a lower prevalence was recorded by Coelho *et al.* (2011) [11] from clinical and subclinical mastitis with an incidence of 16.6% and 46.6% respectively and Ali *et al.* (2018) [3] recorded *hla* gene in 24% isolates.

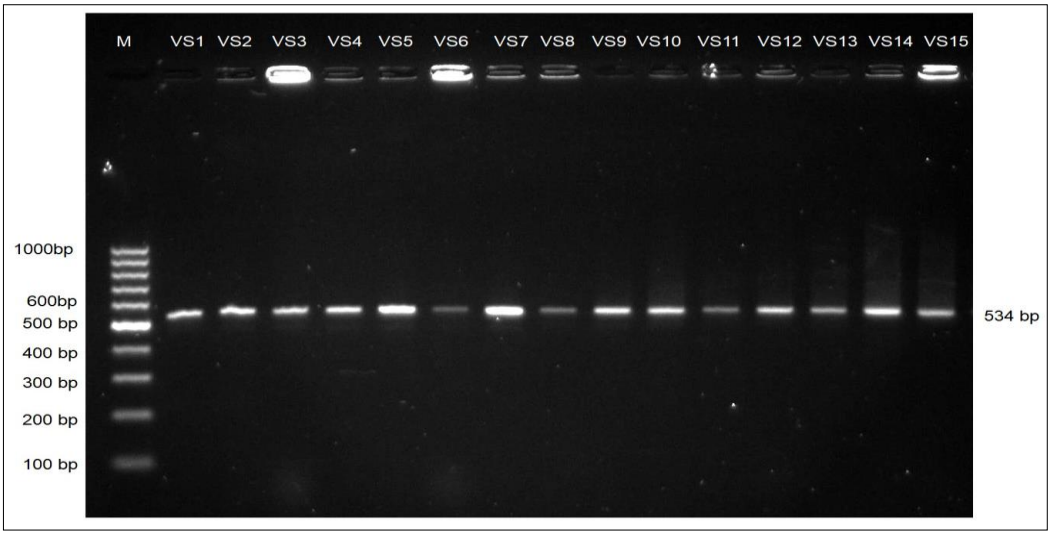

Fig 2: PCR amplicons of *hla* gene of *S. aureus* isolates, M-Molecular marker (100 bp)

The *hlb* gene prevalence in our study was in accordance with Yang *et al.* (2012) [35] reported 85% *S. aureus* isolates in clinical mastitic milk of buffaloes carrying *hlb* gene. However, lower prevalence of *hlb* gene was found by many workers *viz*. Jarraud *et al.* (2002) [20] reported that only 10% isolates showed positive for *hlb* gene among 198 human clinical isolates, Coelho *et al.* (2011) [11] reported *hlb* gene in 16% isolates.

In the current study, we found a comparative high prevalence of *hla* gene than *hlb* gene which was in agreement with the study of Ali *et al*. (2018) [3] who detected haemolysin type A in clinical and subclinical mastitis with an incidence of 71.4% and 70% respectively, while haemolysin type B was detected in clinical and subclinical mastitis with an incidence of 28.5% and 40% respectively. In our study, most of the isolates (80%) were recorded to carry both genes which is contrast to Fei *et al*. (2011) [15] who detected 27 (20.93%) of 129 *S. aureus* isolates from mastitic milk obtained from buffalo with both *hla* and *hlb* genes.

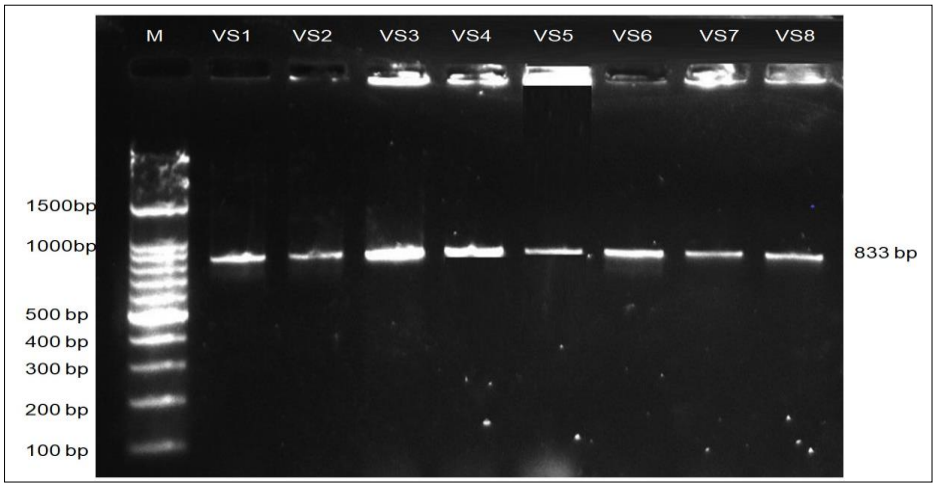

Fig 3: Agarose gel electrophoresis of *hlb* gene of *S. aureus* isolates, M-Molecular marker (100 bp)

Conclusion

In present study no association was found between presence of haemolysin genes and their phenotypic expression. This may be due to either silencing of *hla* and *hlb* genes or these genes are not expressed resulting in non-haemolytic phenotypes. The variations in hemolysis pattern of *S. aureus* indicated that diversity existed among isolates in regards to hemolysis property. It may warrant the urgency to study the genes related with hemolysis factor.

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