International Journal of Advanced Biochemistry Research

ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2024; SP-8(1): 738-742
www.biochemjournal.com
Received: 25-10-2023
Accepted: 28-11-2023

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Detection of *hla* and *hlb* gene in *Staphylococcus aureus* isolates from buffaloes with subclinical mastitis

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DOI: https://doi.org/10.33545/26174693.2024.v8.i1Sj.417

Abstract
Staphylococcus aureus (*S. aureus*) is a spherical, non-motile, gram-positive non-spore forming bacterium which plays a critical role to cause subclinical mastitis in buffaloes. In present study 84 milk samples were collected from Hanumangarh city, 41 *S. aureus* isolate were isolated with a recovery of 48.80%. All 41 samples were genotypically confirmed by 23S rRNA based ribotyping method. All the 41 isolates were typable for haemolysin genes where *hla* gene was detected in 37 (90.24%) and *hlb* gene in 34 (82.92%) isolates. On sheep blood agar 25 isolates (60.97%) produced complete hemolysis, four (9.75%) incomplete hemolysis both complete and incomplete by four (9.75%) while eight (19.51%) isolates did not produce any hemolysis. Hence *S. aureus* isolates obtained from buffaloes with subclinical mastitis showed higher percentage of haemolysin both genotypically and phenotypically.

Keywords: Buffaloes, *hla* and *hlb* gene, *Staphylococcus aureus*, subclinical mastitis

Introduction
A subclinical mastitis is a non-observable form of mastitis which is characterized by having no visible sign in mammary gland but decrease in milk production, change in milk composition and sudden increase in somatic cell count is observed (Guidry, 2007; Argaw, 2016) [17, 5]. Hemolysis is the lysis of erythrocytes by hemolysin toxin produced by *S. aureus*. Hemolysin toxins are classified into four types: alpha, beta, gamma, and delta. *S. aureus* has an important feature called hemolysis, which plays a key part in the pathological process of the ailments caused by this pathogen. In the development of subcutaneous infections in animals, α-hemolysin is a prominent causal agent (Patel *et al*., 1987) [25]. Hemolysis is an instance in which erythrocytes are lysed due to the production of α- and β-toxin (hemolysins) by *S. aureus* (Foster, 1996) [15]. The most extensively investigated pore-forming exotoxin, alpha-hemolysin, is a heptameric protein with a monomer size of 34 kD. It generates gaps in cell membranes and lyses erythrocytes, mast cells, and thrombocytes from several animal species, most notably rabbit (Haslinger *et al*., 2003) [18]. It is controlled by the *hla* gene. Beta-haemolysin is a 39 kD protein exotoxin enzyme with Mg++ dependent sphingomyelinase-C activity that degrades sphingomyelin in erythrocyte, leukocyte, neuron, and other tissue cell membranes (Gow and Robinson, 1969; Bernheimer *et al*., 1974) [16, 6]. So, this study was designed to detect the *hla* and *hlb* gene in *Staphylococcus aureus* isolates from buffaloes with subclinical mastitis.

Materials and Methods
Sample collection and Isolation of *S. aureus*
A total of 84 visually normal milk samples in the range of 5-10 ml were obtained from buffaloes (collected from all quarters) pertaining to various farmers in and around Hanumangarh (Rajasthan). The samples were immediately sent to the laboratory on ice in sterilised test tubes for further processing. Methodlogy of Cowan and Steel (1975) [12] and Quinn *et al*. (1994) [26] were used for Isolation and identification of *S. aureus.*

Hemolysin production

Sheep blood agar medium was used to test hemolysin properties of bacterial isolates and the reactions were categorized as complete-hemolysis, partial-hemolysis, both or none.

Genotypic confirmation of *Staphylococcus aureus*

Isolation of bacterial DNA was done as per the method by Nachimuttu *et al.* (2001) [24] with little modifications. The genotypic confirmation was done through 23S rRNA ribotyping as per the method by Straub *et al.* (1999) [29] using the primers as mentioned in table-1. The master mix was prepared by mixing GENETAQ Green Master Mix (2X) 12.5 µl, primers (25 pM/µl) 0.5 µl each, DNA template 3.0 µl and Nuclease free water to make 25.0 µl. The PCR cycle included pre denaturation at 95 °C for 5 min, 37 cycle of three steps (denaturation at 94 °C for 40s, annealing at 64 °C for 60s and extension at 72 °C for 75s) and final extension at 72 °C for 7 m

Amplification of *hla* and *hlb* gene

For the amplification of the *hla* and *hlb* genes, Booth *et al.* (2001) [8] 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 rRNAribotyping was done. The amplification was done in thermocycler with initial denaturation at 94 °C for 30 sec and followed by 30 cycles primer annealing 53 °C for 1 min, primer extension 72 °C for 30 sec. Final extension was done at 72 °C for 7 min and reaction is terminated by holding at 4 °C. The PCR products 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 (5 µl) were run along with 100 bp. DNA ladder (Invitrogen) and amplicons were visualized under UVP Gel Doc Bioimaging System.

Results and Discussion

Genotypic confirmation of *Staphylococcus aureus*

In this study all 41 isolates were confirmed by 23S rRNA based ribotyping method, Similar genotypic method of *S. aureus* identification have been employed by many workers (Sanjiv *et al*., 2008; Upadhyay *et al*., 2010; Bhati *et al*., 2016) [27, 30, 7] with the same primers as described by Straub *et al*., (1999) [29].

Hemolysis on sheep blood agar

Lysis of erythrocytes by hemolysin toxin released from *S. aureus*, is called hemolysis. Production of hemolysins is another important phenotypic property of *S. aureus* isolates. Different workers have examined *S. aureus* isolated from variety of sources and found variations in the production of hemolysin which is greatly affected by the environmental conditions. *Staphylococcus aureus* produces three types of hemolysins namely α, β and d designated in order of their discovery (Elek and Levy, 1950) [13].

In the present study all of the 41 *S. aureus* isolates from buffalo subclinical mastitic milk were streaked on sheep blood agar and incubated at 37 °C for 18 to 24 hrs to record hemolysis. Out of 41 isolates, 25 isolates (60.97%) produced complete hemolysis, four (9.75%) incomplete hemolysis both complete and incomplete by four (9.75%) while eight (19.51%) isolates did not produce any hemolysis. The results are shown in Table 2.

The higher prevalence of beta hemolysis was also reported by Anosike *et al.* (2019) [4] for 15 *S. aureus* isolates they reported, 2 (13.33%) isolates exhibited alpha hemolysis on blood agar, unlike others (86.66%) that were beta hemolytic from the wounds of hospital patients. Islam *et al.* (2007) [20] also recorded more than 86.3% *S. aureus* from cattle showing incomplete hemolysis. Jahan *et al.* (2015) [21] reported (100%) isolates showed β-hemolysis on blood agar media enriched with 5% sheep blood, which was contrary to our result.

In the present study eight (19.51%) isolates did not produce any hemolysis and were considered ahemolytic. Many other workers have recorded a variable percentage of ahaemolytic *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 these observations, Boerlin *et al.* (2003) [9] did not detect incomplete hemolysis on the blood agar plate by *S. aureus* isolates. Many of the workers (Sanjiv and Kataria, 2007; Morandi *et al.*, 2009; Upadhyay and Kataria, 2010; Helal *et al.*, 2015) [28, 23, 30, 19] did not record any ahemolytic isolates in their studies.

A double zone of both complete and incomplete hemolysis together was reported in five (16.66%) isolates in our study. Both types of hemolysis were also reported by Wald *et al.* (2019) [32] they found double-zone hemolysis (both complete and incomplete hemolysis) in 61% field isolates and 37% isolates were classified as β-hemolytic. No hemolysis was found in two per cent of *S. aureus* isolates.

Similar results were reported by Younis *et al.* (2000) [34], Wald *et al.* (2019) [32] where they found α-hemolysis, β-hemolysis, both α and β types of hemolysis and no hemolysis with *S. aureus* isolates from cattle milk samples.in contrast to this result, Upadhyay and Kataria (2010) [30], Khichar and Kataria (2015) [22], Jahan *et al.* (2015) [21] and Bhati *et al.* (2016) [7] reported all the isolates from cattle milk to show hemolysis on sheep blood agar.

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.

***hla* and *hlb* gene**

Hemolysins, alpha and beta are governed by *hla* and *hlb* genes, respectively and are among important virulence factors in *S. aureus*. Lysis of erythrocytes by hemolysin toxin released from *S. aureus*, is called haemolysis. There are several types of hemolysin toxins namely alpha, beta, gamma and delta. They are cytolytic pore-forming toxins to a variety of host cells which not only lyse cells but depress host immune response also. In the present investigation, 41 *S. aureus* isolates from buffaloes with subclinical mastitis were subjected to PCR amplification of these two (*hla* and *hlb*) hemolytic genes. In our study all isolates were typable.

Seven isolates produced amplicons of 534 bp (Fig.2 and Table. 3) and were considered *hla* gene-positive isolates, four isolates produced amplicon of 833 bp (Fig.3 and Table. 3) which was considered as an isolate with *hlb* gene and 30 (73.17%) isolates were typable for both of the genes.

In the present study, the *hla* gene was detected in 37 (90.24%) *S. aureus* isolates. The similar results were reported by Acosta *et al.*, (2018) [1], where they recorded 88% *S. aureus* isolates to harbour *hla* gene. Low prevalence (24%) for *hla* gene was reported shown by Coelho *et al.*, (2011) [11] which was much lower than that in present study.

In the present investigation 34 (82.92%) *S. aureus* isolates harboured with *hlb* gene. Similarly, Yang *et al.* (2012) [32] reported 85% *S. aureus* isolates from milk from clinical mastitic buffaloes with *hlb* gene. Contrary to our result low prevalence was reported by Ali *et al.* (2018) [3] from clinical mastitis and subclinical mastitis with an incidence of 16.6% and 46.6% respectively.

Table 1: Primers used for detection of genes in *S.aureus* isolates from buffaloes with subclinical mastitis

S No.	Target Gene	Primer sequence	Size (bp)	Reference
1	23S rRNA	F: 5´-ACGGAGTTACAAAGGACGAC-3´ R: 5´-AGCTCAGCCTTAACGAGTAC-3´	1250	(Straub *et al.*, 1999) [29]
2	*hla*	F-5'-GGTTTAGCCTGGCCTTC-3' R-5'-CATCACGAACTCGTTCG-3'	534	(Booth *et al.*, 2001) [8]
3	*hlb*	F-5'-GCCAAAGCCGAATCTAAG-3' R-5'-CGCATATACATCCCATGGC-3'	833	(Booth *et al.*, 2001) [8]

Table 2: Hemolysis by *S aureus* isolates obtained from buffalo with subclinical mastitis

S. No.	Isolates (Total)	Type of hemolysis
1.	PB2, PB3, PB4, PB5, PB6, PB7, PB8, PB9, PB11, PB14, PB15, PB16, PB18, PB20, PB22, PB23, PB24, PB26, PB27,PB28, PB29, PB 34, PB38,PB39, PB40. (25)	Complete
2.	PB1, PB25, PB36, PB41. (4)	Incomplete
3.	PB21, PB30, PB32, PB33. (4)	Both complete and incomplete
4.	PB10, PB12, PB13, PB17, PB19, PB31, PB35, PB37 (8)	No hemolysis

Table 3: Detection of hemolysin genes (*hla* and *hlb*) in *S. aureus* isolates from buffaloes with subclinical mastitis

S. No.	Gene type	Isolate numbers	Total isolates	Amplicon size (bp)
1.	*Hla*	PB3, PB16, PB20, PB25, PB26, PB35,PB38	7 (17.07%)	534
2.	*Hlb*	PB17, PB27, PB30, PB37	4 (9.75%)	833
3.	Both *hla* and *hlb*	PB1, PB2, PB4, PB5, PB6, PB7, PB8, PB9, PB10, PB11, PB12, PB13, PB14, PB15, PB18, PB19, PB21, PB22, PB23, PB24, PB28, PB29, PB31, PB32, PB33, PB34, PB36, PB39, PB40, PB41.	30 (73.17%)	534, 833

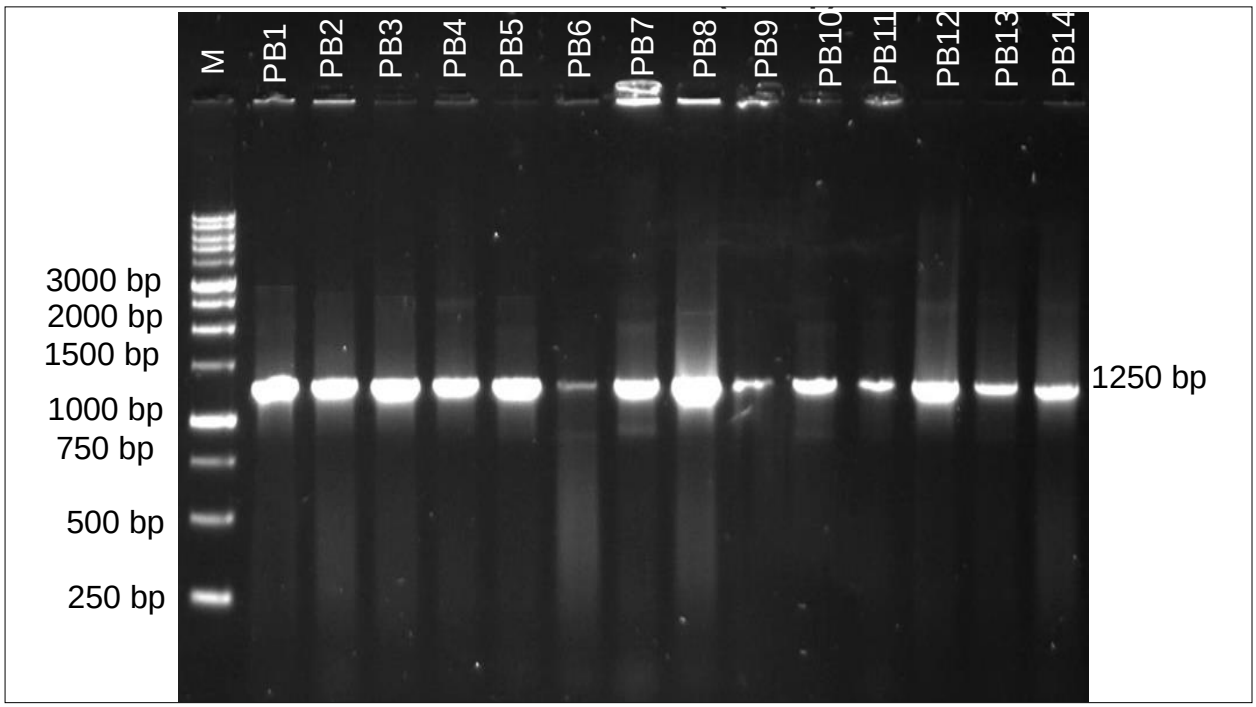

Fig 1: PCR amplicons of 23S rRNA ribotyping of *S. aureus* isolates

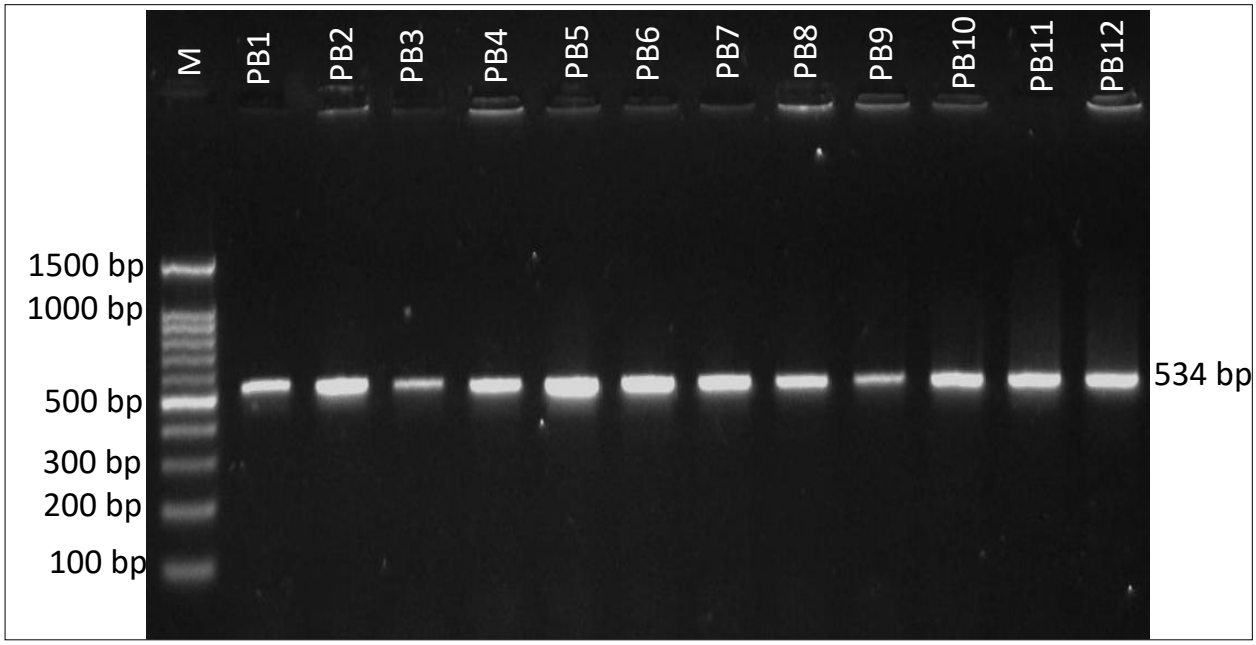

Fig 2: Agarose gel electrophoresis of amplicons of *hla* gene *S. aureus* isolated from buffaloes with subclinical mastitis. M-Molecular marker (100 bp)

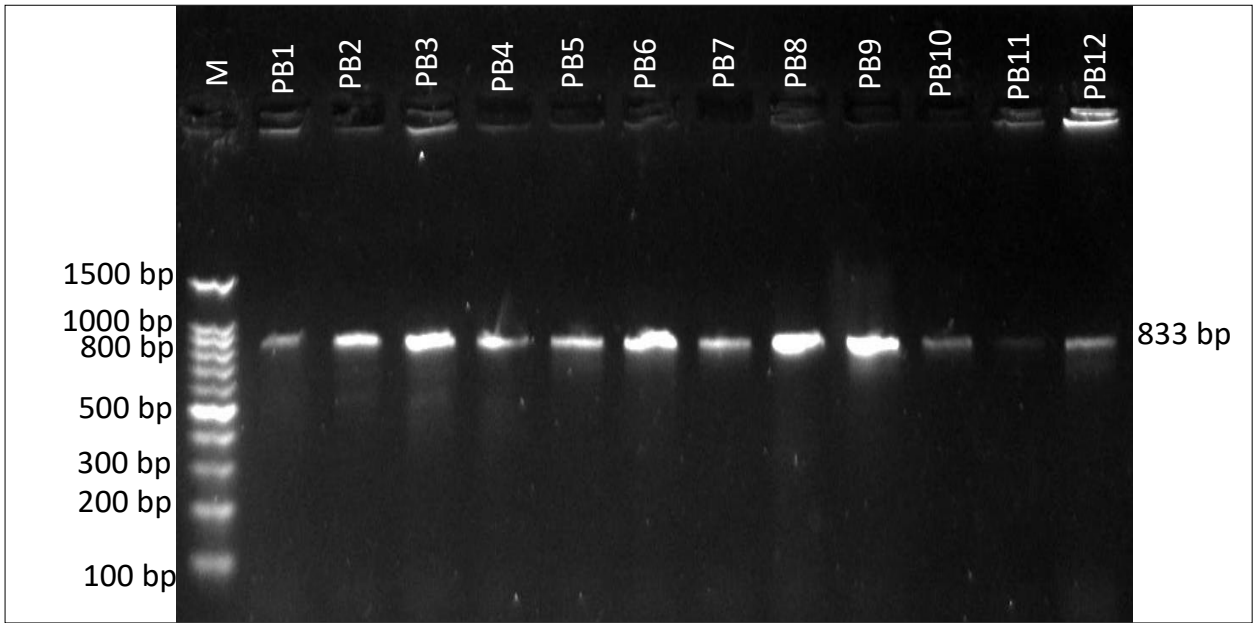

Fig 3: Agarose gel electrophoresis of amplicons of *hlb* gene *S. aureus* isolated from buffaloes with subclinical mastitis. M-Molecular marker (100 bp)

Conclusion

In the current investigation, no link was established between the existence of haemolysin genes and their phenotypic manifestation. This could be because the *hla* and *hlb* genes are silenced or not expressed, resulting in non-haemolytic phenotypes. *S. aureus* hemolysis patterns varied, indicating that isolates differed in hemolysis properties. It may justify the need to study the genes associated with the hemolysis factor.

Acknowledgement

The authors express their gratitude to Department of Veterinary Microbiology and Biotechnology, College of Veterinary and Animal Science, Bikaner, Rajasthan University of Veterinary and Animal Sciences (RAJUVAS), Bikaner.

Conflict of interest

There are no conflicts of interest to declare by any of the authors.

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