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Molecular Detection of Some Virulence Genes of *Staphylococcus aureus* Isolates Associated with Bovine Mastitis in Arid and Semi-Arid Regions of India

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ABSTRACT

Keywords

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Staphylococcus aureus (*S. aureus*) is one of the most common causes of contagious bovine mastitis and its different virulence factors involved in the pathogenesis are well documented worldwide. The present study was conducted in 107 isolates of *S. aureus* recovered from mastitic milk (n=51), udder surfaces (n=35) and milkers' hands (n=21) for detection and characterization of genes encoding for hemolysins (*hla*, *hly* and *hld*), leucocidin (*luk-PV*), surface proteins (*clfA*, *clfB*, *fnbA*, *fnbB*) and penicillin resistance (*blaZ*) by multiplex PCR. All the isolates were positive for *blaZ*, *hla*, *hly* and *clfA* genes while 94.4%, 85%, 63.6%, 44.9% and 19.6% isolates carried *clfB*, *hly*, *fnbA*, *fnbB* and *luk-PV* genes, respectively. Mastitic milk isolates showed higher frequency of *clfB* (96.07%) and *hly* (88.2%) genes, udder isolates for *fnbB* (48.6%) and *luk-PV* (42.9%) genes while isolates of human origin showed higher occurrence of *fnbA* (66.7%) genes. An overall high prevalence of hemolysins, surface proteins, adhesins, and penicillin resistance genes was observed. A little association was seen between presence of hemolysin and *blaZ* genes and their phenotypic expression. The recovery of penicillin resistant *S. aureus* strains from bovine mastitis and close human contacts in India is of concern in clinical management of mastitis.

Introduction

Bovine mastitis is an economically important multi etiological disease and *Staphylococcus aureus* is the predominant contagious

mastitogen in India (Gandhale *et al.*, 2017) and worldwide (Hanon, 2017). The pathogenicity of *S. aureus* is related to the presence of wide variety of virulence factors enabling adherence, colonisation, invasion of

the mammary cells, evasion of the immune defence mechanism and survival in the host environment (Memon *et al.*, 2013).

The initial attachment of *S. aureus* to epithelial cells of the teat canal depends on the interaction of bacterial surface proteins called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), such as clumping factors A and B (*clfA* and *clfB*) and fibronectin-binding proteins A and B (*fnbA* and *fnbB*), with host fibrinogen and fibronectin proteins located in the basement membrane, around myoepithelial cells and fibroblasts (Ni Eidhin *et al.*, 198; Wann *et al.*, 2000). *Staphylococcus aureus* *clfA* and *clfB* and *fnbA* and *fnbB* proteins are also involved in the evasion of host immune responses due to their ability to bind fibrinogen on the *S. aureus* cell wall thereby inhibiting deposition of or access to opsonins to the pathogen (Higgins *et al.*, 2006).

Staphylococcus aureus secretes several cytolytic toxins, among them are alpha, beta, and delta hemolysins and Panton-Valentine leukocidin (PVL). Cytolytic toxins form β -barrel pores in the plasma membrane and cause leakage of the cell's content and lysis of the target cell (Kaneko and Kamio, 2004).

Beta and alpha hemolysins are the most important in pathogenesis of the intramammary infections (Park *et al.*, 2004). The presence of PVL positive *S. aureus* strains from human and its clinical implications have been reported (Cirkovic *et al.*, 2012) but scanty information is available about its presence in bovine mastitic milk and udders especially in India.

Although there are many reasons which compromise antibiotic treatment of *S. aureus* mastitis of which resistance of bacteria toward antibiotics is the most important one. Beta-lactam compounds such as penicillin

continues to be one of the most frequently used drugs in veterinary medicine (Pitkala *et al.*, 2007). Two primary resistance mechanisms to beta-lactams are there in *Staphylococcus* spp.: the expression of beta-lactamase enzymes encoded by the *blaZ* gene, and production of the penicillin-binding protein 2a (PBP2a) encoded by the *mecA* gene, which is coding resistance against almost all beta-lactam antibiotics which are widely used in mastitis treatment (Benic *et al.*, 2012).

The strains of *S. aureus* have been reported to vary in pathogenicity due to diversion in expression of genes and showed differences in severity of mastitis (Haveri *et al.*, 2007; Capurro *et al.*, 2010).

Hence the present study was carried out for molecular detection of genes coding alpha, beta, and delta hemolysins and Panton-Valentine leukocidin (PVL), clumping factors A and B (*clfA* and *clfB*), fibronectin-binding proteins A and B (*fnbA* and *fnbB*) and penicillin resistance (*blaZ*) in 107 *S. aureus* isolates from bovine mastitis and close human contacts in arid and semi-arid regions of India.

Materials and Methods

Ethical approval

This study was conducted following approval by the research committee and guidelines of Institutional animal ethics committee were followed.

Isolation, identification and genotypic confirmation of *S. aureus* isolates

A total number of 107 *S. aureus* isolates comprising of 51 isolates from mastitic milk, 35 isolates from udder surface swabs and 21 from milkers' hands swabs collected from seven different farms in arid and semi-arid

regions of Rajasthan, India were included in the study. The isolates were primarily identified by Gram staining, catalase test, coagulase test and fermentation of mannitol salt agar (Markey *et al.*, 2013) and genotypically confirmed by 23S rRNA gene based ribotyping (Straub *et al.*, 1999) and *nuc* gene amplification as per Brakstad *et al.*, (1992) as described earlier (Bhati *et al.*, 2018).

Hemolysins characterization

The patterns of hemolysis by *S. aureus* isolates were examined by streaking on sheep blood agar plates and incubating at 37 °C for 24 h (Quinn *et al.*, 2000).

The hemolysins were identified using following criteria: complete zone of hemolysis with unclear edges for α -hemolysin and incomplete zone of hemolysis which after incubation at 4 °C overnight became complete with sharp edges, for beta hemolysin (Quinn *et al.*, 2000; da Silva *et al.*, 2005).

Beta-lactamase activity (Acidimetric method)

Hydrolysis of the β -lactam ring generates a carboxyl group, acidifying unbuffered systems. The resulting acidity can be tested in tubes by the method described by (Livermore and Brown, 2001) in which 2 ml of 0.5% (w/v) aqueous phenol red solution was diluted with 16.6ml distilled water, and 1.2g of benzylpenicillin was added to it.

The pH was adjusted to 8.5 with 1M NaOH and 100 μ l of the resulting solution (violet in color) was distributed into microtitre wells and inoculated with bacteria from culture plates to produce dense suspensions.

Development of yellow colour within 5 min indicated β -lactamase activity. Positive and negative controls were run in parallel.

Molecular detection of *S. aureus* virulence genes by PCR

Bacterial culture was grown overnight and DNA was isolated (Nachimuttu *et al.*, 2001) and DNA quantification was carried out by spectrophotometric measurements (Sambrook *et al.*, 1989). Three sets of multiplex PCR were designed for genes having similar annealing temperatures with slight modifications: Set A for *blaZ*, *fnbA* and *fnbB*; Set B for *luk-PV*, *hld* and *clfB* genes and Set C for *clfA*, *hla* and *hly*. The PCR mixture for each set of multiplex PCR consisted of 5.0 μ l 5X Go Taq® Flexi buffer, 3.0 μ l MgCl₂ (25mM), 1.0 μ l dNTP mix (25mM each), 0.2 μ l Taq DNA polymerase (5U/ μ l), 3.0 μ l DNA template (30 ng/ μ l), primers sequences and concentration as described in Table 1 and nuclease free water to make a total volume of 25 μ l. Amplification was carried out in a Veriti thermal cycler (Applied biosystem) using protocol as mentioned in Table 2. Amplified PCR products (5 μ L) were separated by electrophoresis in 1.2% agarose gel stained by ethidium bromide and visualized under a UV transilluminator (ENDURO GDS).

Results and Discussion

In the present study an overall recovery rate of *S. aureus* was 54.3% with highest prevalence of 63.8% in mastitic milk samples followed by 53% (udder surface) and 41.2% (milkers' hands), as described earlier (Bhati *et al.*, 2018). Of the 107 isolates, five (4.7%) isolates produced complete (alpha) haemolysis, 58 (54.2%) produced partial (beta) haemolysis and three (2.8%) exhibited both complete and partial hemolysis on blood agar. When plates with partial haemolysis were subjected to further incubation at 4 °C, 54 isolates (93.1%) showed turning of partial hemolysis to complete hemolysis (hot-cold lysis) whereas other four isolates (6.9%) did not show hot-cold lysis. Hence a total of 66 (61.7%) *S.*

aureus isolates were hemolytic and 41 (38.3%) did not produce haemolysis on blood agar. From mastitic milk samples, 62.7% (32/51) isolates were haemolytic followed by from milkers' hands being 61.9% (13/21) and 60.1% (21/35) haemolytic isolates from udder.

All the 107 *S. aureus* isolates were subjected to PCR amplification for hemolysin genes (*hla*, *hly* and *hld*) and Panton-Valentine leukocidin (*luk-PV*) gene using specific primers. The *hla* and *hld* genes were detected in all the isolates (100%) producing a single amplicon of 534 bp and 111 bp respectively (Figure 1 and 2). The detection rate of *hly* gene was 85% with 88.2% mastitic milk isolates carrying *hly* gene followed by isolates from udder (82.9%) and 81% from milkers' hands (Table 3). The present investigation detected very low prevalence of *luk-PV* gene in only 21(19.6%) isolates yielding amplicon of 433 bp while 80.4% isolates were negative for it. Highest prevalence of *luk-PV* gene was recorded in isolates from udder 15/35 (42.9%) followed by isolates from milkers' hands (14.3%) and only 5.9% isolates from mastitic milk were positive for it.

The prevalence of adherence associated genes, *clfA*, *clfB*, *fnbA* and *fnbB* was 100%, 94.4%, 63.6% and 44.9% respectively. Higher frequency of *clfB* gene in mastitic milk isolates (96.07%), *fnbA* gene in milkers' hands isolates (66.7%) and *fnbB* gene in 48.6% udder isolates was recorded. While the beta-lactamase activity was exhibited by 78 (72.9%) isolates but 100% isolates were positive for *blaZ* gene (Figure 3). Highest beta lactamase activity was recorded among mastitic milk isolates (80.4%) while more than 60% isolates from udder and milkers' hands showed this activity.

The present study revealed a high prevalence of bovine mastitis due to *S. aureus* in different farms and greater frequency of isolation of *S.*

aureus from mastitic milk, udder surfaces and close human contacts. This also suggests the lack of proper management practices of maintaining animal health as well hygiene of the farm and farm workers in arid and semi arid regions of India. The pathogenicity of *S. aureus* is due to the presence of different virulence factors leading to invasion, colonization and infection of host (Soares *et al.*, 2017) and diversity in these virulence factors produced by *S. aureus* strains isolated from bovine mastitis has been reported (Capurro *et al.*, 2010).

Staphylococcal haemolysins are identified as important virulence factors that contribute for bacterial invasion and to escape from the host immune response. In our study, most of *S. aureus* strains showed haemolytic activity on blood agar which is consistent with the results obtained by other authors (Capurro *et al.*, 2010; Kot *et al.*, 2013). The higher prevalence of partial (β) hemolysis in *S. aureus* strains observed in present study is similar to the findings of Islam *et al.*, (2007) and Yadav *et al.*, (2015a) who reported 89.3% and 62.5% of *S. aureus* strains of bovine mastitic origin to produce incomplete hemolysis. Some workers have reported a higher prevalence of complete or alpha hemolysis in *S. aureus* isolates from various sources (Wang *et al.*, 2011; Younis *et al.*, 2017) which is contrary to present findings. The variations recorded in hemolysis pattern of *S. aureus* isolates from various sources in present or previous studies indicate diversity among isolates with regards to hemolysis property.

By PCR amplification of the genes encoding hemolysins of *S. aureus* with specific primers it could be observed that *hla* and *hld* genes were present in all isolates while 85% isolates were positive for *hly* gene. In the present study 91 (85%) isolates were positive for all the three hemolysin genes *hla*, *hly* and *hld* and 16 isolates did not carry *hly* gene but were

positive for *hla* and *hld* genes. Of these 16 *hly* negative isolates, 10 were phenotypically ahaemolytic on sheep blood agar while remaining six was found to be of haemolytic phenotype. This may be due to expression of α or δ toxins which either alone or synergistically produced hemolysis on blood agar plate. Out of 41 phenotypically ahaemolytic strains, 10 did not carry *hly* gene but were positive for *hla* and *hld* genes while the remaining 31 isolates were found to carry *hla*, *hly* and *hld* genes. This may be due to either silencing of *hla* and *hly* genes or these genes are not expressed resulting in haemolytic phenotypes. Hence, a little association was observed between hemolytic genotypes and their phenotypic expression. This is similar to the findings of Wang *et al.*, (2011) and Ariyanti *et al.*, (2011) who concluded that the hemolysin genotypes of *S. aureus* and their phenotypic expression do not correlate well. However, Younis *et al.*, (2017) recorded prevalence of *hla* genes (90.90%) and *hly* (85.45%) gene which correlated with their phenotypic hemolytic activity on blood agar plates which is in contrast to present findings.

In the present investigation the prevalence of *hly* gene recorded was higher in bovine strains (86%) than human strains (81%). Likewise, Delgado *et al.*, (2011) observed *hly* gene more common in bovine isolates (80%) than in human isolates (20%). In contrast Moraveji *et al.*, (2014) found the *hly* gene in 3 (15%) out of 20 isolates collected from bovines and 8 (40%) out of 20 isolates collected from humans. The *hly* gene present in *S. aureus* isolates of human origin (55%), bovine origin (16%) and from food sources (18%) was also reported by Salasia *et al.*, (2011) contrary to present observation. Some workers reported higher percentage of isolates with *hly* gene than *hla* gene (Yang *et al.*, 2015; Wang *et al.*, 2016). Although the presence of *luk-PV* genes in *S. aureus* isolates from bovine mastitis is

still insufficiently explored but similar low prevalence of *luk-PV* gene has also been reported by other workers from bovine mastitis *i.e.* 2.7% by Yang *et al.*, (2015), 7.1% by Awad *et al.*, (2017) and 24.8% by Hoque *et al.*, (2018). In contrast, a higher prevalence (65.6%) of *luk-PV* gene was observed by El-Sayed *et al.*, (2015) in *S. aureus* isolates from clinical mastitis in cattle and buffaloes. In the present study 21% bovine strains were positive for *luk-PV* gene as compared to 14.3% of human strains which is contrary to the study of Pajic *et al.*, (2014) who recorded *luk-PV* gene in 6.67% and 63.63% of bovine and human isolates of *S. aureus*, respectively.

Regarding the occurrence of genes encoding for *S. aureus* surface proteins involved in adhesion, results obtained in this study have shown similarities and few differences with previous research. The *clfA* gene was detected in 100% of the isolates, which is similar to findings of Felipe *et al.*, (2017) and Baloch *et al.*, (2018). Klein *et al.*, (2012) detected 91.8% and Xu *et al.*, (2015) found 85.7% *clfB* positive strains from bovine mastitis similar to present study while Felipe *et al.*, (2017) and Zhang *et al.*, (2018) detected *clfB* gene in 100% isolates. As compared to present study, a lower prevalence of *clfA* and *clfB* genes was reported by Memon *et al.*, (2013), He *et al.*, (2014) and de Almeida *et al.*, (2017). The present study revealed single amplicon of 1000 bp for *clfA* gene while polymorphic band patterns *viz.* 950 (3.7%), 1000 (70.0%) and 1100bp (20.5%) were observed by Kumar *et al.*, (2011). Further Reinoso *et al.*, (2008), Karahan *et al.*, (2011) and Yadav *et al.*, (2015b) observed two different amplicons of 900 and 1000bp sizes showing polymorphism. The present investigation revealed presence of fibronectin binding proteins, *fnbA* (63.6%) and *fnbB* (44.9%), in *S. aureus* isolates which play important role in adherence to bovine mammary gland cells leading to infectious mastitis.

Table.1 Nucleotide sequences used as primers

S. No	Gene	Primer sequence (5' to 3')	Primer concentration	Amplicon size	Reference
1.	<i>hla</i>	F-5' GGT TTA GCC TGG CCT TC 3'	4 pM/μl	534 bp	Booth <i>et al.</i> , (2001)
		R-5' CAT CAC GAA CTC GTT CG 3'	4 pM/μl		
2.	<i>hlb</i>	F-5' GCC AAA GCC GAA TCT AAG 3'	4 pM/μl	833 bp	Booth <i>et al.</i> (2001)
		R-5' CGC ATA TAC ATC CCA TGG C 3'	4 pM/μl		
3.	<i>hld</i>	F-5'- AAG AAT TTT TAT CTT AAT TAA GGA AGG AGT G-3'	2 pM/μl	111bp	Jarraud <i>et al.</i> (2002)
		R-5'-TTA GTG AAT TTG TTC ACT GTG TCG A-3'	2 pM/μl		
4.	<i>luk-PV</i>	F-5'- -ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A-3'	2 pM/μl	433 bp	Lina <i>et al.</i> (1999)
		R-5' -GCA TCA AST GTA TTG GAT AGC AAA AGC- 3'.	2 pM/μl		
5.	<i>clfA</i>	F-5'-GGC TTC AGT GCT TGT AGG-3'	4 pM/μl	1000bp	Stephan <i>et al.</i> (2001)
		R-5'-TTT TCA GGG TCA ATA TAA GC-3'	4 pM/μl		
6.	<i>clfB</i>	F-5'-ACA TCA GTA ATA GTA GGG GGC AAC-3'	2 pM/μl	205bp	Tristan <i>et al.</i> (2003)
		R-5'-TTC GCA CTG TTT GTG TTT GCA C-3'	2 pM/μl		
7.	<i>fnbA</i>	F-5'-GCG GAG ATC AAA GAC AA-3'	4 pM/μl	1279 bp	Nashev <i>et al.</i> (2004)
		R-5'-CCA TCT ATA GCT GTG TGG-3'	4 pM/μl		
8.	<i>fnbB</i>	F-5'-GGA GAA GGA ATT AAG GCG-3'	4 pM/μl	812 bp	Nashev <i>et al.</i> (2004)
		R-5'-GCC GTC GCC TTG AGC GT-3'	4 pM/μl		
9.	<i>blaZ</i>	F-5'-AAG AGA TTT GCC TAT GCT TC-3'	4 pM/μl	517bp	Sawant <i>et al.</i> (2009)
		R-5'-GCT TGA CCA CTT TTA TCA GC-3'	4 pM/μl		

Table.2 Cycling conditions used in PCR

PCR Type	Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Multiple x Set A	<i>blaZ</i> , <i>fnbA</i> , <i>fnbB</i>	94°C 5 min	94°C 1 min	50°C 1 min	72°C 1 min	30	72°C 7 min
Multiple x Set B	<i>luk-PV</i> , <i>hld</i> , <i>clfB</i>	94°C 5 min	94°C 1 min	55°C 1 min	72°C 1 min	30	72°C 7 min
Multiple x Set C	<i>clfA</i> , <i>hla</i> , <i>hnb</i>	94°C 5 min	94°C 1 min	50°C 1 min	72°C 1 min	30	72°C 7 min

Fig.1 PCR amplicons of *clfA*, *hla*, *hnb* genes of *S. aureus* isolates using DNA ladder (M) of IKbp

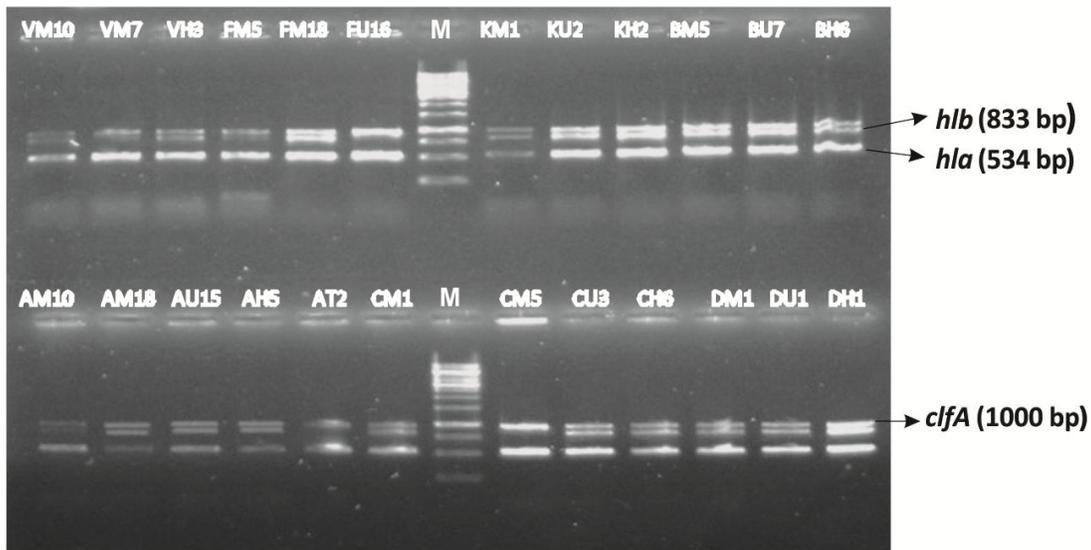


Fig.2 PCR amplicons of *clfB*, *hld*, *luk-PV* genes of *S. aureus* isolates using DNA ladder (M) of I00 bp

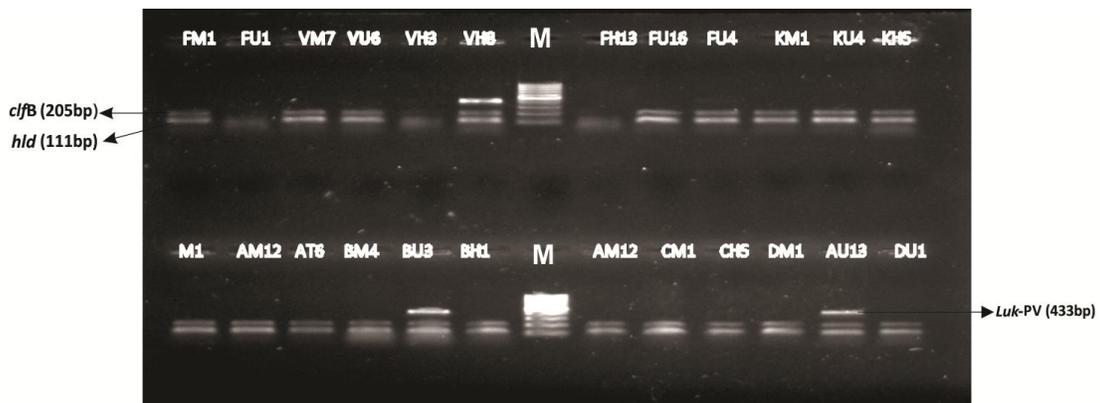


Fig.3 PCR amplicons of *fnbA*, *fnbB*, *blaZ* genes of *S. aureus* isolates using DNA ladder (M) of 500 bp

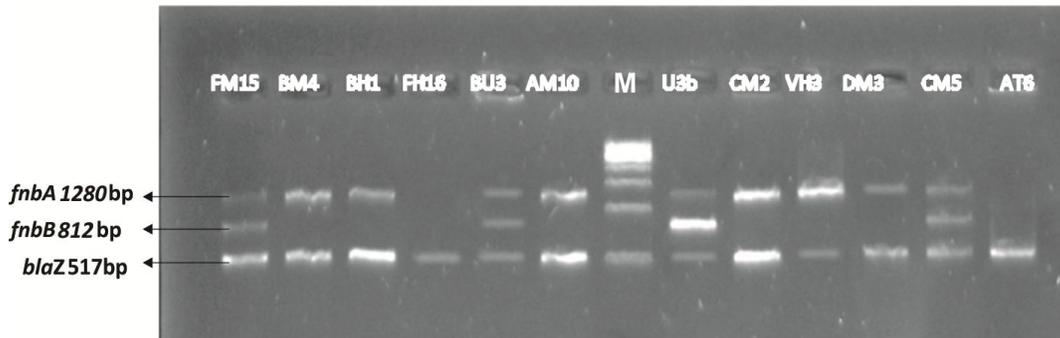


Table.3 Prevalence of different virulence factors in *S. aureus* isolates

Source of sample	Prevalence (%)									
	<i>fnbA</i>	<i>fnbB</i>	<i>clfA</i>	<i>clfB</i>	<i>hla</i>	<i>hlb</i>	<i>hld</i>	<i>LuK-PV</i>	β -lactamase production	<i>blaZ</i>
Mastitic milk (n-51)	32 (62.7)	21 (41.2)	51 (100)	49 (96.1)	51 (100)	45 (88.2)	51 (100)	03 (5.9)	41 (80.4)	51 (100)
Udder swabs (n-35)	22 (62.9)	17 (48.6)	35 (100)	32 (91.4)	35 (100)	29 (82.9)	35 (100)	15 (42.9)	23 (65.7)	35 (100)
Milkers hands swabs (n-21)	14 (66.7)	10 (47.6)	21 (100)	20 (95.2)	21 (100)	17 (81)	21 (100)	03 (14.3)	14 (66.7)	21 (100)
Total n=107	68 (63.6)	48 (44.9)	107 (100)	101 (94.4)	107 (100)	91 (85)	107 (100)	21 (19.6)	78 (72.9)	107 (100)

The prevalence of *fnbA* gene has been reported as high as 100% in *S. aureus* isolates from intramammary infections by workers from different study areas (Yang *et al.*, 2012; Felipe *et al.*, 2017; Baloch *et al.*, 2018) to as low as 27.3% by Soares *et al.*, (2017). Further, *fnbA* gene was not detected in any strain from bovine subclinical mastitis by Memon *et al.*, (2013) and He *et al.*, (2014). As compared to present study a higher percentage of *S. aureus* isolates were detected with *fnbB* gene by other workers from bovine mastitis *i.e.* 80% by Wang *et al.*, (2016) 76.3% by Felipe *et al.*, (2017); 78.2% by Soares *et al.*, (2017) and 85% by Zhang *et al.*, (2018) while none of the investigated *S. aureus* strains harbouring *fnbB*

gene was reported by Yang *et al.*, (2012). These variations have been suggested to depend either on the specific geographic region or, alternatively, on methodological differences. Khoramian *et al.*, (2015) studied 215 *S. aureus* strains collected from human and dairy cow's infections and reported the prevalence of *fnbA* gene among the bovine isolates significantly higher than those in the human isolates which is contrary to present findings where the prevalence of *fnbA* gene among the human isolates was higher.

Prevalence of penicillin resistance in staphylococci causing animal diseases is most commonly due to the *blaZ* gene encoding for

penicillinases (beta lactamase). In contrast to present study, lower beta-lactamase production in 55.9% and 9% of the isolates from clinical mastitis was reported by Turutologu *et al.*, (2006) and Capurro *et al.*, (2010) respectively. Bagcigil *et al.*, (2012) identified 78 beta-lactamase positive isolates out of 147 isolates with positive *blaZ* gene while Marques *et al.*, (2017) reported 100% beta lactamase producing *S. aureus* isolates from bovine mastitis.

The present study detected *blaZ* gene in all the isolates which is similar to findings of Yang *et al.*, (2015) who detected *blaZ* gene in 94.6% of penicillin resistant *S. aureus* isolates from bovine mastitis cases. Our result of all the isolates carrying *blaZ* gene but only 78 (72.9%) isolates exhibiting beta-lactamase activity is in contrast to the results of Robles *et al.*, (2014) who reported that several *S. aureus* isolates from bovine mastitis did not harbor the *blaZ* gene but phenotypic tests showed beta-lactamase activity. Hence, beta-lactamase phenotype could be result of expression of more than one gene and more than one mechanism is responsible for beta-lactam resistance other than the expression of *blaZ* gene. As reported by Robles *et al.*, (2014) it was observed that PCR detection of *blaZ* gene had a low association with all phenotypic tests and there could be a strong possibility that beta-lactamase production was herd-dependent.

In conclusion, the high percentage of hemolysin, *clfA* and *clfB* producing strains obtained in this work suggest an important role of these virulence factors in the pathogenesis of bovine mastitis in the farms. The strains showed diversity in the pattern of the virulence gene profiles. The presence of genes and their phenotypic expression did not correlate well in this study. Further the development of resistance towards beta-lactam antibiotics is of concern in the arid and

semiarid regions of India which may pose problems in the treatment of clinical cases. Considerable pathogenic strains were obtained from milk handlers which may be either transmitted to or contracted from infected cattle which require further research. This also indicates the requirement to educate the farmers towards mastitis management.

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