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Virulent Gene Characterization of Methicillin Sensitive *Staphylococcus aureus* (MSSA) and Methicillin Resistant *Staphylococcus aureus* (MRSA) from Bovine Mastitis

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A B S T R A C T

The objective of the study was to characterize the virulence gene profile of MSSA and MRSA isolated from bovine mastitis. A total of 160 mastitic milk samples were collected from different regions of Jammu. Following different molecular techniques, a total of 36(37.5%) isolates were confirmed as *S. aureus*. These 36 *S. aureus* isolates were inoculated on ORSA to determine the phenotypic MRSA isolates. Only 20(55.5%) isolates were confirmed to be MRSA phenotypically. All 36 isolates were subjected to *mecA* gene amplification by PCR. Only 6 isolates were *mecA* gene positive hence MRSA. Both MRSA and MSSA isolates were screened for virulence genes viz. coagulase (*coa*), staphylococcal protein A(*spa*), clumping factor (*clfA*), haemolysin A(*hla*) and enterotoxins (*eta* &*etb*). The *coa* gene was present in 35(97.2%), *spa* in 35(97.2%), *clfA* in 35(97.2%), *hla* in 22(61.1%) and *eta* &*etb* in none of the isolates.

Introduction

Staphylococcus aureus present worldwide is a gram positive coccus, facultative anaerobe habitually positive for catalase and nitrate reduction and is coagulase variable which can be positive or negative. This organism can get transmitted from animals to humans through contamination of skin lesions while in contact with the affected animals, other possible vehicle including milkers hand and milking equipment is responsible for transmission of organism between animals. Due to its virulence factors like hyluronidase, protease, lipase, nuclease; coagulase; Clumping factors (Clf A); fibrinogen binding proteins (Fnbp A); Protein A; Panton-Valentine leucocidin (PVL); Staphylococcal Enterotoxin (SE) A to

SEEand SEG to SEU; Exfoliative toxin (A & B); Toxic shock syndrometoxin-1 (TSST-1); alpha and beta haemolysins,*S. aureus* exhibits wide range of diseases commonest of which is Mastitis thus deteriorating the milk quality (Gulzar *et al.,* 2018, Hamid *et al.,* 2017).Owing to mounting resistance to antibiotics, it is difficult to control virulent strains of *S. aureus* from causing mastitis posing greater threat to dairy industry (Hamid *et al.,* 2017).

Soon after the resistance of *S. aureus* to penicillin antibiotic, in late 1950s β lactamase resistant methicillin was introduced. However soon after introduction of Methicillin, the *S. aureus* developed resistance to it. Strains of *S. aureus* resistant to β-lactam antibiotics are known as methicillin-resistant *S. aureus* (MRSA) (Robinson and Enright, 2003). Methicillin resistance is caused by the acquisition of *mecA* gene. This produces an alternative penicillin binding protein PBP2a, which has lower affinity for β-lactam antibiotics (Vanderhaeghen *et al.,* 2010). The *mecA* gene is a part of a large mobile genetic element, the staphylococcal cassette chromosome mec (SCCmec). Studies have shown that MRSA strains possess more toxin genes as compared to MSSA strains (Cai Youngwe *et al.,* 2007). MRSA strains isolated from different geographical areas have shown to possess distinct toxin gene profiles (Cai Youngwe *et al.,* 2007). The bovine MRSA may be transmitted to humans and may contribute to outbreaks in human populations (Lee, 2003). Therefore, monitoring the dissemination of virulence determinants and drug resistance genes among *S. aureus* isolates from bovine mastitis has become indispensable. The identification and characterization of virulence factors of *S. aureus* causing bovine mastitis will enhance our understanding of the pathogenesis of intra-mammary infection and may pave way for reduction in losses incurred to the dairy industry. Hence, this study was aimed for molecular characterization of virulent genes of *S. aureus* isolates obtained from mastitic milk of various dairy farms in Jammu.

Materials and Methods

Sample collection

This study was based on a total of 160 milk samples (80 cows and 80 buffaloes) collected from clinical as well as subclinical mastitis cases. Before sampling, the udders were inspected for externally observed pathological condition. Each milk sample was collected aseptically in a sterile screw-capped bottle from each mammary gland after washing with water and cleaning the teats with cotton soaked in 70% ethanol. The samples were immediately taken to the laboratory for bacteriological analysis. The various steps pertaining before characterizing the virulence genes are given in Diagram 1 and the sequence of the primers for PCR amplification specific to different genes of *Staphylococcus aureus* are given in Table 1.

Molecular detection of virulence genes

All the *S. aureus* isolates were screened for following virulence genes.

Detection of coagulase (*coa*) gene

The PCR amplification was performed in 25 µl in 0.2 ml thin walled PCR tubes (Eppendorf, Germany). The PCR mixture contained a final concentration of 2.5µl of 10× coloured buffer, 3 mM $MgCl_2$, 1 µM concentration of each primer (Table 1), 0.5 mM concentrations of each 2′-deoxynucleoside 5′-triphosphate and 2U of Taq DNA polymerase (Promega, USA). The amplification cycles in Mastercycler Gradient Thermal cycler (Eppendorf, Germany) consisted of 94°C for 45s, followed by 30

cycles of 94°C for 20 s, 57°C for 15 s and 70°C for 15 s and final extension at 72°C for 2min. Positive control DNA samples maintained in the laboratory were included in the PCR. Sterile distilled water was used as negative control.

Detection of staphylococcal protein A(*spa*) gene

The PCR amplification was performed in 25 µl in 0.2 ml thin walled PCR tubes (Eppendorf, Germany) as per Montesinos *et al.,* (2002). Positive control DNA samples maintained in the laboratory were included in the PCR. Sterile distilled water was used as negative control.

Detection of clumping factor A(*clfA)*gene

The PCR amplification was performed in 25 µl in 0.2 ml thin walled PCR tubes (Eppendorf, Germany) as per Stephan *et al.*, (2000). Positive control DNA samples maintained in the laboratory were included in the PCR. Sterile distilled water was used as negative control.

Detection of staphylococcal enterotoxin genes (*sea and seb*)

The PCR amplification was performed in 25 µl in 0.2 ml thin walled PCR tubes (Eppendorf, Germany) as per Monday *et al.*, (1999). Positive control DNA samples maintained in the laboratory were included in the PCR. Sterile distilled water was used as negative control.

Detection of alpha- haemolysin(*hla*)

The PCR amplification was performed in 25 µl in 0.2 ml thin walled PCR tubes (Eppendorf, Germany) as per Booth *et al.*, (2001). Positive control DNA samples maintained in the laboratory were included in the PCR. Sterile distilled water was used as negative control.

Results and Discussion

Results of the study have been presented below with suitable figures and tables wherever necessary isolates were inoculated on ORSA to determine the phenotypic MRSA isolates. Fromtotal 36 confirmed *S. aureus* isolates only 20(55.5%) isolates were confirmed to be MRSA phenotypically as shown in Fig. 1 and all 36 isolates were subjected to *mecA* gene amplification by PCRonly 6(16.6%) isolates were confirmed to be methicillin resistant *staphylococcus aureus* (MRSA). The PCR product appeared as a single band with a size close to the expected size of 533 bp as indicated in Fig. 2.

The relative distribution of all the studied virulence genes, in methicillin- resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) isolates is depicted in Table 1.

Molecular detection of virulence genes coagulase gene (*coa*)

Out of 36 confirmed *S. aureus,* 35(97.2%) amplified the *coa* gene specific product as depicted in Fig. 2. Out of 30 MSSA isolates, 29(96.6%) were coagulase positive strains and all MRSA isolates 6(100%) were coagulase positive. PCR amplified fragments as shown in Fig. 3.

For *S. aureus* the production of coagulase is considered as an important criterion for its identification and the detection of the gene responsible for coagulase production is often used for its characterization (Ahmadi *et al.*, 2010). However, presence of *coa* (coagulase) gene is not species specific as *S. aureus* isolates deficient of this gene have also been confirmed (Kobayashi *et al.,* 1995; Sanjiv *et*

al., 2008). In our investigation, 100% and 96.6% of isolates of MRSA and MSSA were coagulase positive respectively. This finding is in agreement with the report by (Kumar *et al.,* 2011; Boerlin *et al.,* 2003; Aarestrup *et al.,* 1999) who also recounted 100% MRSA and 88.2% MSSA isolates *coa* gene positive.

Staphylococcal protein A(*spa*) gene

Out of 36 confirmed *S. aureus*, 35(97.2%) were positive for Staphylococcal protein A and showed polymorphisim or variable PCR amplified fragments as shown in Fig. 4. Out of 30 MSSA isolates, 29(96.6%) were spa gene positive whereas all MRSA isolates were positive for *spa* gene.

The *spa* gene is composed of functionally distinct regions, i.e. Fc binding region, X-region and at C-terminus. The X-region of the spa gene includes a variable number of 24-bp repeats (Kuzma *et al.,* 2003; Frenay *et al.,* 1994) and because of this the *spa* genes have been the most widely used markers for molecular typing.

In our study, 100% of MRSA and 96.6% of MSSA isolates were *spa* gene positive which is in agreement with Marques *et al.,* 2013 reported presence of spa gene in all of the isolates from bovine mastitis, showing variable amplicon sizes with 300 bp being the prevalent size.

Contrary to the results in the present study, uniform amplicons of 300 bp size were obtained by Suleiman *et al.,* 2012 in 20 isolates of *S. aureus* from subclinical bovine mastitis. The *S. aureus* isolates have also been reported to be *spa* gene-deficient by Shakeri *et al.,* 2010. Enterotoxins of *S. aureus* play an important role in modulating the host immune response and contribute to maintain a suitable environment for its colonization (Omoe *et al.,* 2003).

Clumping factor (*clf*A)gene

Out of 36 confirmed *S. aureus*, 35(97.2%) isolates were clumping factor(clfA) positive and showed a single amplicon with a size of approximately 980 bp as shown in Fig. 5. All MRSA isolates carried *clfA* gene while as one MSSA isolate lacked *clfA* gene.

Clumping factor A (*clfA*) gene is an important adhesion factor of *S. aureus* that facilitate its binding via soluble or immobilized fibrinogen. The *clfA* has also has the ability to inhibit phagocytosis by human PMNL which may explain its importance in *S. aureus* virulence in a variety of animal models of infection (Higgins *et al.,* 2006). The screening of *S. aureus* isolates showed that the *clfA* gene was present in 100% and 99.6% of MRSA and MSSA isolates, respectively. Majority of the previous studies reported 100% correlation between *clfA* and *coa* gene in bovine *S. aureus* isolates (Akineden *et al.,* 2001; Stephan *et al.,* 2001; Salasia *et al.,* 2004 and Reinoso *et al.,* 2008) which is in concordance with our results.

Alpha-haemolysin (*hla*) gene

Out of 36 confirmed *S. aureus*, 22(61.1%) were alpha haemolysin (*hla*) positive and showed a single amplicon with a size of approximately 535 bp as shown in Fig. 6. Among MSSA isolates, only 18 out of 30 (60%) carried *hla* gene whereas only 4(66.6%) out of six MRSA isolates were positive for this gene.

Haemolysin is recognized as a potential virulence factor of *S. aureus*. According to Cifrian *et al.,* 1996 interaction between alpha and beta haemolysin increases both adherence to bovine mammary epithelial cells and the proliferation of *S. aureus.* Haemolysin toxins include α-, β- and δ-toxin act on cell membranes of RBC causing hemolysis (Table 2).

Table.1 The sequence of the primers for PCR amplification specific to different genes of *Staphylococcus aureus*

Primer name	Primer sequence (5′– 3′)	Reference	Size (bp)
***nuc*-1 F**	GCGATTGATGGTGATACGGTT	Louie *et al.*, (2002)	**270**
***nuc*-2 R**	AGCCAAGCCTTGACGAACTAAAGC		
Staur 4	ACGGAGTTACAAAGGACGAC	Straub *et al.*, (1999)	**1250**
Staur 6	AGCTCAGCCTTAACGAGTAC		
***mecA* F**	AAAATCGATGGTAAAGGTTGGC	Louie *et al.*, (2002)	**530**
***mecA* R**	AGTTCTGCAGTACCGGATTTGC		
***coa* F**	ATAGAGATGCTGGTACAGG	Hookey *et al.*, (1998)	**variable**
***coa* R**	GCTTCCGATTGTTCGATGC		
***spa* F**	TCAAGCACCAAAAGAGGAAGA	Montesinos *et al.*, (2002)	**variable**
***spa* R**	GTTTAACGACATGTACTCCGTTG		
***sea* F**	GCAGGGAACAGCTTTAGGC	Monday *et al.*, (1999)	**520**
***sea* R**	GTTCTGTAGAAGTATGAAACACG		
***seb* F**	ACATGTAATTTTGATATTCGCACTG		**643**
***seb* R**	TGCAGGCATCATGTCATACCA		
hla F	GGTTTAGCCTGGCCTTC	Booth *et al.*, (2001)	**535**
hla R	**CATCACGAACTCGTTCG**		

Table.2 Absolute and relative distribution of staphylococcal virulence genes, coagulase (*coa*), staphylococcal protein A (*spa*), clumping factor (*clfA*), haemolysin (*hla*) and enterotoxins (*sea* and *seb*) according to methicillin- resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* isolate (MSSA)

Gene	Total n=36 n(%)	MSSA n=30 n (%)	MRSA n=06 n (%)
One or more	35 (97.22)	29(96.60)	6(100)
coa	35 (97.22)	29(96.6)	6(100)
spA	35 (97.22)	29(96.6)	6(100)
clfA	35 (97.22)	29(96.6)	6(100)
hla	22 (61.11)	18(60)	4(66.66)
sea	0	0	0
seb	0	0	0

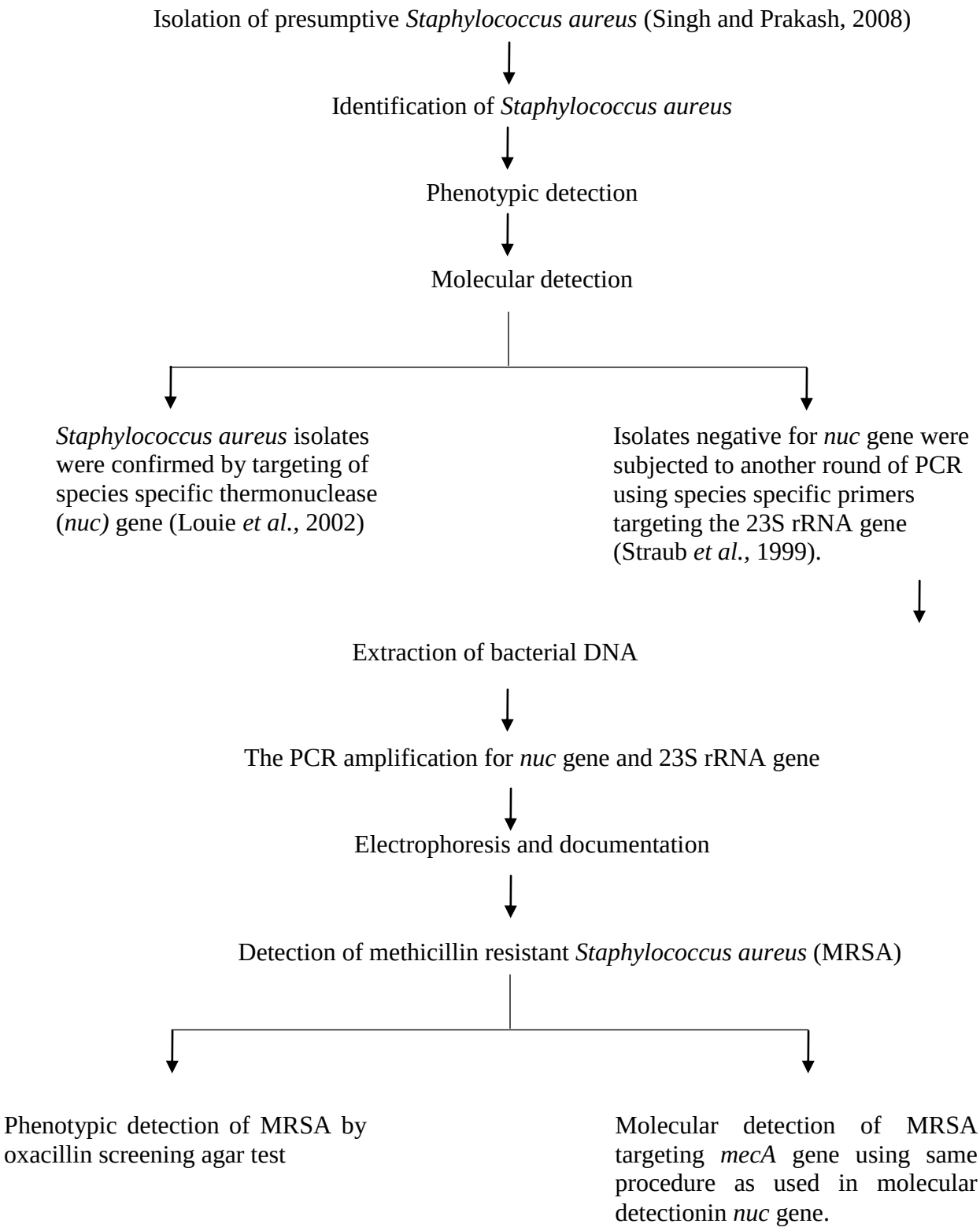

Diagram.1 Steps pertaining before characterizing virulence genes

Fig.1 Typical blue colonies of *S. aureus* on ORSA agar showing phenotypic MRSA isolates

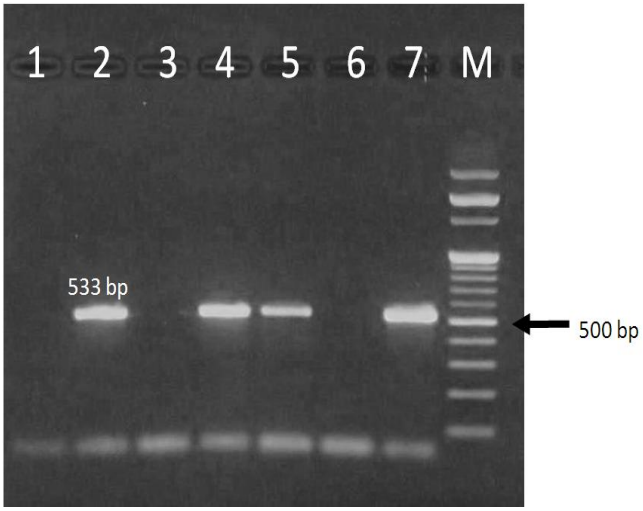

Fig.2 PCR amplification of *MECA* gene of *S. aureus* isolates

Lane M - 100bp DNA ladder. Lane 1,3 - Negative isolates.
Lane 2,4,5 - Positive isolates. Lane 6 - Negative control.
Lane 7 - Positive control.

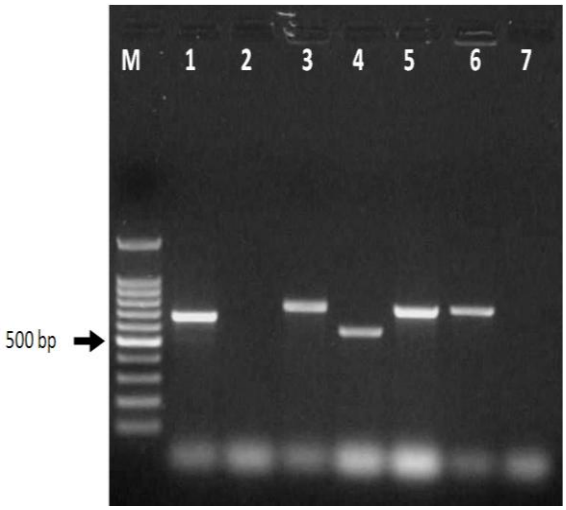

Fig.3 PCR amplification of coagulase gene (*coa*) of *S. aureus* isolates.

Lane M - 100 bp DNA ladder. Lane 1 - Positive control.
Lane 2 - Negative control. Lane 3,4,6 - Positive isolates.
Lane 5,7 - Negative isolate

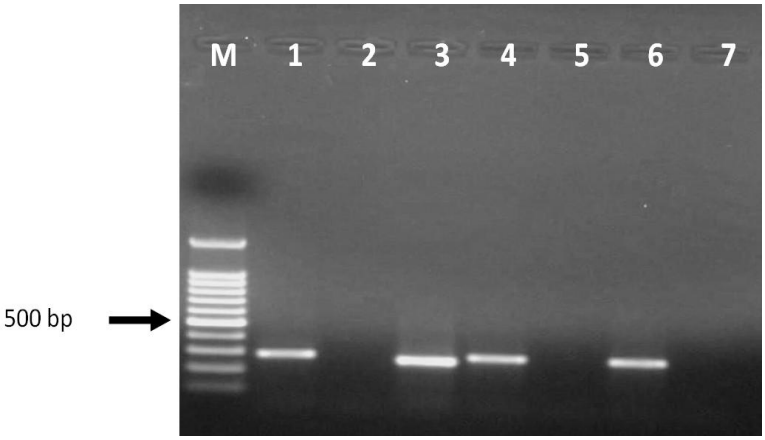

Fig.4 PCR Amplification of staphylococcal protein a gene*(spa)* of *S. aureus* Isolates

Lane M - 100 bp DNA ladder. Lane 1 - Positive control.
Lane 2 - Negative control. Lane 3,4,6 - Positive isolates.
Lane 5,7 - Negative isolate.

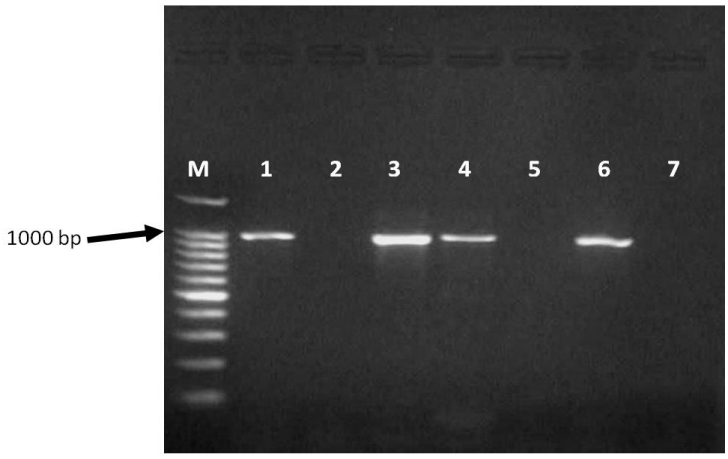

Fig.5 PCR amplification of clumping factor*(clfa)* gene of *S. aureus* isolates

Lane M - 100 bp DNA ladder. Lane 1 - Positive control.
Lane 2 - Negative control. Lane 3,4,6 - Positive isolates.
Lane 5,7 - Negative isolate

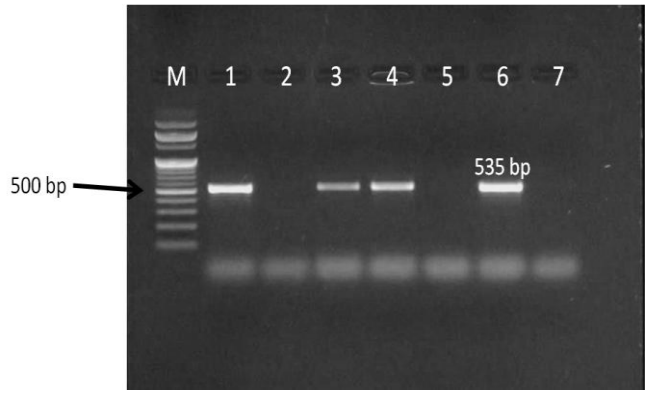

Fig.6 PCR amplification of alpha haemolysin (*hla*) gene of *S. aureus* isolates

Lane M - 100 bp DNA ladder. Lane 1 - Positive control.
Lane 2 - Negative control. Lane 3,4,6 - Positive isolates.
Lane 5,7 - Negative isolate

They can damage platelets, cause lysosome destruction, ischemia and necrosis of the body. In the present investigation, 66.6% of MRSA isolates and 60% of MSSA isolates were *hla* gene positive which is in contrast with Kumar *et al.,* (2011) and Da Silva *et al.,* (2005) as they found 100% and 85% of the isolates positive for *hla* gene. But our results are in agreement with Aarestrup *et al.,* 1999 as he found 56% of the isolates *hlb* gene positive. The remaining *S. aureus* isolates that were negative for *hla* may either have lost *hla* gene or the ability to express it or carried a variant gene and failed to express haemolytic activity in *vitro* as demonstrated by Aarestrup *et al.,* 1999.

Enterotoxin A and enterotoxin B(*eta* and *etb*)

None of the *S. aureus* isolate carried either enterotoxin A (*eta*) or enterotoxin B (*etb*) gene. The main interest of studying enterotoxins, resides in their ability to cause food poisoning. The major enterotoxin gene present in mastitic *S. aureus* isolates include *Sea-See, Seh, Seg, Sei, Sej,Seo* and Tsst-1(Stephan *et al.,* 2001; Akineden *et al.,* 2001, Zschöck *et al.,* 2004).

In the present study, *S. aureus* isolates were screened for the presence of *sea* and *seb* but none of the isolates were positive for these enterotoxins. Previously, researchers have reported variability in the prevalence of enterotoxin genotypes among farms and countries (Akinedin *et al.,* 2001; Gomez *et al.,* 2007; Da Silva *et al.,* 2005). The recovery of the enterotoxin genes in the present study was in agreement with the results of other studies (Oliveira *et al.,* 2011; Fueyo *et al.,* 2005). The possible reason may be that the researchers reported greater prevalence of newly enterotoxin genes (*Seg* to *Seq*), compared with classical enterotoxin genes (*Sea* to *See*).

The results of the present study may differ from previous investigations because of different clinical characteristics of mastitis or because more recently identified enterotoxins were not investigated. It is also possible that there is variability in the occurrence of enterotoxin genes among geographical regions.

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