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
SEE and 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 MgCl₂, 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. From total 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 PCR only 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
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 coagene positive.

**Staphylococcal protein A (spa) gene**

Out of 36 confirmed *S. aureus*, 35(97.2%) were positive for Staphylococcal protein A and showed polymorphism 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 (clfA) 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*   | ACGGAGTTACAAAGGACG       | Straub *et al.* (1999) | 1250      |
| *Staur 6*   | AGCTCAGCCTTAACGAGTAC     |           |           |
| *mecA F*    | AAAATCGATGGTGTAAGGTTG     | Louie *et al.* (2002) | 530       |
| *mecA R*    | AGTCTGCGATACCGGATTTGC    |           |           |
| *coa F*     | ATAGAGATGCTGGTGACGAGG     | Hookey *et al.* (1998) | variable |
| *coa R*     | GCTTCCGATTGTTCTGATG       |           |           |
| *spa F*     | TCAAGCACCACACAGGAGGAAGA   | Montesinos *et al.* (2002) | variable |
| *spa R*     | GTTTAACGACATGTACTCCTGGTG  |           |           |
| *sea F*     | GCAGGGAACAGCCTTAGG       | Monday *et al.* (1999) | 520       |
| *sea R*     | GTTCTGTAGAAGTGAACACG     |           |           |
| *seb F*     | ACATGTAATTTTGTATTCGACTG  |           | 643       |
| *seb R*     | TGCAGGCTACATGTACATACCA   |           |           |
| *hla F*     | GGTITTAGCCTGAGCCTTC      | Booth *et al.* (2001) | 535       |
| *hla R*     | CATCACGAAACTCGTTC       |           |           |

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             |
Isolation of presumptive *Staphylococcus aureus* (Singh and Prakash, 2008)

Identification of *Staphylococcus aureus*

Phenotypic detection

Molecular detection

*Staphylococcus aureus* isolates were confirmed by targeting of species specific thermonuclease (*nuc*) gene (Louie *et al.*, 2002)

Isolates negative for *nuc* gene were subjected to another round of PCR using species specific primers targeting the 23S rRNA gene (Straub *et al.*, 1999).

Extraction of bacterial DNA

The PCR amplification for *nuc* gene and 23S rRNA gene

Electrophoresis and documentation

Detection of methicillin resistant *Staphylococcus aureus* (MRSA)

Phenotypic detection of MRSA by oxacillin screening agar test

Molecular detection of MRSA targeting *mecA* gene using same procedure as used in molecular detection in *nuc* gene.

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

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

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

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

| Lane | Description                        |
|------|------------------------------------|
| M    | 100 bp DNA ladder.                 |
| 1    | Positive control.                  |
| 2    | Negative control.                  |
| 3, 4, 6 | Positive isolates.             |
| 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 (Akineden 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 Sej), 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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How to cite this article:

Hamid Shah, Mohd Altaf Bhat, Burhan Nabi, Anil Taku and Gulzar Ahmed Badroo. 2020. Virulent Gene Characterization of Methicillin Sensitive Staphylococcus aureus (MSSA) and Methicillin Resistant Staphylococcus aureus (MRSA) from Bovine Mastitis. Int.J.Curr.Microbiol.App.Sci. 9(06): 3471-3482. doi: https://doi.org/10.20546/ijcmas.2020.906.409