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Molecular characterisation of virulence genes in *Staphylococcus aureus* associated with clinical bovine mastitis

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Abstract

Staphylococcus aureus is the most frequently isolated pathogen from bovine mastitis including subclinical, clinical, and chronic infections. Virulence factors possessed by S. aureus aid in causing infection and inflammation by producing toxins and proteins, which are responsible for the pathogenesis of the disease. In the current study out of 51 animals presented with clinical mastitis, S. aureus was isolated from the milk of 18 animals. S. aureus was confirmed by genus and species level identification using polymerase chain reaction. Molecular characterization of selected virulence genes including thermonuclease (nuc) and Panton Valentine Leucocidin (PVL) was performed in all the S. aureus isolates. Presence of nuc gene was observed in all the isolates (100 %) of S. aureus. No isolates were found to be positive for the presence of PVL gene. Profiling the virulence genes is an important tool for epidemiological studies of mastitis, which can be employed for the prevention and control of the disease.

Keywords: *Mastitis, Staphylococcus aureus, virulence genes*

Mastitis can be defined as a complex disease which involves interaction between the host anatomy and physiology, different causative pathogens and environmental factors such as animal husbandry, hygiene and sanitation. In dairy industry worldwide, *S. aureus* is said to be the most common causative organism responsible for bovine mastitis (Miles *et al*., 1992). The infected quarter of affected cows is considered to be the main reservoir for *S. aureus* infection in the herd. The different factors like evasion of immune mechanism of host, invasion and infection of host tissue, spread of bacteria and acquisition of the required nutrients by the pathogenic organism like *S. aureus* could be attributed to its virulence factors (Haveri *et al.,* 2005). The *nuc* gene is

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said to be ubiquitous in all organisms belonging to genus *Staphylococcus*. It is said that regardless of the thermonuclease activity all *Staphylococcus* spp., except *S. scuiri*, possess *nuc* gene (Sasaki *et* al., 2007). Panton Valentine Leucocidin is a cytotoxin, which causes disruption of cell membranes creating pores on polymorphonuclear neutrophils (Kaneko and Kameo 2004). Panton Valentine Leucocidin positive *S. aureus* was found to be responsible for causing mastitis in many countries and it was observed that even though PVL acts strongly on human polymorphonuclear cells, weak activity was observed on bovine neutrophils too (Peton and Le Loir, 2014).

The study of the important virulence factors aids in the identification of their role in bovine mastitis. Each specific virulence factors have role in each stage and type of infection, and it is observed that not all strains of *S. aureus* possess the same virulence factors. Hence, study of virulence factors in *S. aureus* is useful in molecular epidemiological studies of bovine clonal types for the effective prevention and control of mastitis (Fitzgerald *et al*., 2000). Hence, this study was envisaged for the molecular detection of virulence genes, *nuc* and *PVL* from clinical mastitis caused by *S. aureus*.

Materials and methods

Milk samples were collected from 51 animals affected with clinical mastitis and subjected to identification of organisms by morphological characterization, colony characteristics on selective media and biochemical reactions (Barrow and Feltham, 1993; Quinn *et al*., 2013). Isolates presumptive of *S. aureus* were further subjected for confirmation by molecular characterisation. The DNA extraction was performed by snap chill method or heat lysis method as described by Vijayakumar and Jose (2021). All the isolates of *S. aureus* obtained in the study were subjected to genotypic characterisation by amplification of 16S rRNA and 23S rRNA genes for molecular confirmation of *Staphylococcus* spp. and *S. aureus* respectively. The presence of selected virulent factor genes *viz. nuc* for thermonuclease and *PVL* for Panton Valentine Leucocidin were determined by polymerase chain reaction (PCR) using specific primers as shown in Table 1. The reagents and chemicals used for the PCR were Emerald Amp Fast PCR master mix (2X PCR Smart mix, Takara, Japan) forward and reverse primer set (100nM/ml, Sigma Aldrich) and sterile nuclease free water. All the primers were reconstituted in sterile nuclease free water to a final concentration of 10 pmol/µl and stored at -20 °C.

The PCR were performed using the programmable S1000 Thermal cycler, BioRad, USA. Polymerase chain reaction was performed in a total volume of 25µl reaction mixture by combining the reagents. The PCR conditions were optimized by setting different time temperature combinations for annealing processes (Tables 2, 3). The combination that gave the best result for amplification was selected for carrying out further PCR.

After completion of PCR, amplified products were subjected to submarine agarose gel electrophoresis.

Results and discussion

In the present study, out of the samples from 51 animals affected with clinical mastitis, 35 samples yielded growth and a total 38 bacterial isolates were obtained wherein, three samples yielded two different types of growth. Out of the 38 organisms isolated during this study, 27 were contagious pathogens (71.05 %) which included 18 isolates of *S*. *aureus* (47.37 %), 6 isolates of coagulase negative staphylococci (15.79 %) and 3 isolates of *Micrococcus* spp. (7.89 %). The environmental pathogens (28.95 %) that were isolated included 11 coliforms, out of which 6 isolates were *E. coli* (15.79 %) and 5 isolates were *Klebsiella* spp. (13.16 %). *Staphylococcus aureus* was isolated as the major pathogen from bovine mastitis cases and the result was in accordance with the findings by Verma *et al*. (2018), and Workineh *et al*. (2002) who reported the prevalence of *S. aureus* as 42.55 per cent and 40.5 per cent, respectively. Studies conducted on bovine clinical mastitis by Rathish *et al.* (2015) in Thrissur district also revealed *S. aureus* to be the most common pathogen isolated. The present study did not concur with the findings of Fadlelmula *et al.* (2009), who found lower prevalence of *S. aureus*

Table 1. Details of primers used in PCR

Organism / Virulence genes	Genes	Primer sequence	Amplicon size (bp)	Reference
Staphylococcus spp.	*16S rRNA*	F: AACTCTGTTATTAGGGAAGAA CA	756	Ciftci *et al*. (2009)
		R: CCACCTTCCTCCGGTTTGTCACC		
Staphylococcus aureus	*23S rRNA*	F: GGA CGA CAT TAG ACG AAT CA	1318	El - Razik *et al*. (2010)
		R: CGG GCA CCT ATT TTC TAT CT		
Thermonuclease	*nuc*	F: GCCAAGCCTTGACGAACTAAAGC	279	Brakstad *et al.* (1992)
		R: GCGATTGATGGTGATACGGTT		
Panton Valentine Leucocidin	*PVL*	F: GCTGGACAAAACTTCTTGGAATAT	85	Pajic *et al.* (2014)
		R:GATAGGACACCAATAAATTCTGGATTG		

Table 2. PCR protocol for the amplification for characterization of *S. aureus*

Sl. No	PCR Programme		Temperature – Time Protocol	
			16*S rRNA*	**23*S rRNA***
1.	Initial Denaturation		94 °C for 5 min	94 °C for 5 min
2.	Denaturation	30 cycles	94 °C for 45 sec	94 °C for 45 sec
3.	Annealing		56.9 °C for 45 sec	55.8 °C for 45 sec
4.	Extension		72 °C for 90 sec	72 °C for 90 sec
5.	Final extension		72 °C for 10 min	72 °C for 10 min
6.	Hold		4 °C Until use	4 °C Until use

Table 3. PCR protocol for the amplification of virulence genes of *S. aureus*

Sl. No	PCR Programme		Temperature - Time Protocol	
			nuc	**PVL**
1.	Initial Denaturation		94 °C for 5 min	95 °C for 5 min
2.	Denaturation	30 cycles	94 °Cfor 1 min	95 °C for 30 sec
3.	Annealing		55 °C for 30 sec	59 °C for 30 sec
4.	Extension		72 °C for 90 sec	72 °C for 60 sec
5.	Final extension		72 °C for 3.5 min	72 °C for 5 min
6.	Hold		4 °C Until use	4 °C Until use

of 9.8 per cent in their study. Jose *et al.* (2021) reported in their studies a lower prevalence of coliforms, *Klebsiella* spp. (7.40 per cent) and *E. coli* (4.47 per cent), isolated from clinical bovine mastitis which was consistent with our study.

Hence, from clinical bovine mastitis cases, 18 isolates of *S. aureus* were identified by biochemical methods and subjected to molecular confirmation by polymerase chain reaction after DNA extraction by snap chill method. This method was used for extraction of bacterial DNA from cases of bovine mastitis by many researchers (Shah *et al*., 2020; Nazir *et al.,* 2017). Lange *et al*. (2015) performed species level identification of *Staphylococci* and concluded that *16S rRNA* sequencing is an accurate method for the identification of staphylococci from bovine mastitis. But due to the lack of heterogenicity in *16S rRNA* gene, it would be difficult to identify and discriminate different *Staphylococcus* species (Petti *et al*., 2008).

Stephan *et al.* (2001) used *23S rRNA* for the species level identification of *S. aureus* from bovine mastitis, and all the 34 isolates could be identified as *S. aureus.* Hence, all the bacterial isolates presumed to be *Staphylococcus* spp. were subjected to PCR by targeting *16S rRNA* and for the species level identification of the isolates, *23S rRNA* was targeted. From clinical bovine mastitis, 18 isolates were identified and confirmed as *S. aureus* respectively (Fig. 1 and Fig. 2).

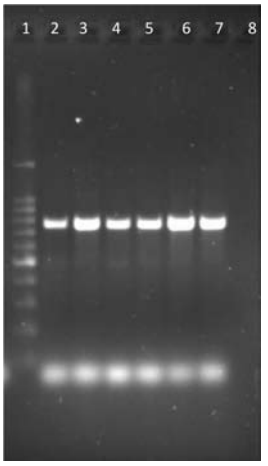

Fig. 1. Agarose gel electrophoresis of *16S rRNA* specific PCR of *Staphylococci* spp.
Lane 1 – Ladder
Lane 7 – Positive control
Lane 8 – Negative control
Lane 2,3,4,5,6,7 – Positive samples (756 bp)

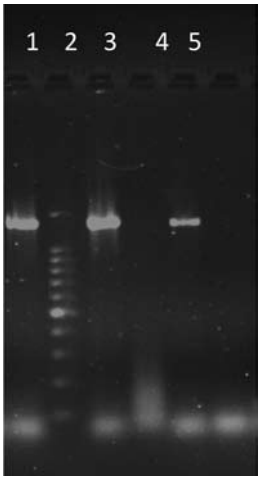

Fig. 2. Agarose gel electrophoresis of *23S rRNA* specific PCR of *S. aureus*
Lane 1,3 – Positive samples (1318 bp)
Lane 2 – Ladder
Lane 4 – Negative control
Lane 5 – Positive control

The virulence factors thermonuclease and Panton Von Leukocidin coded by *nuc* and *PVL* respectively were screened. The *nuc* gene is commonly employed for the species level identification of *S. aureus*. The positive amplicon size of 279 bp for *nuc* gene was detected in all the 18 isolates (100 per cent) of *S. aureus* (Fig.3). This was in accordance with many studies wherein, *nuc* gene was used for

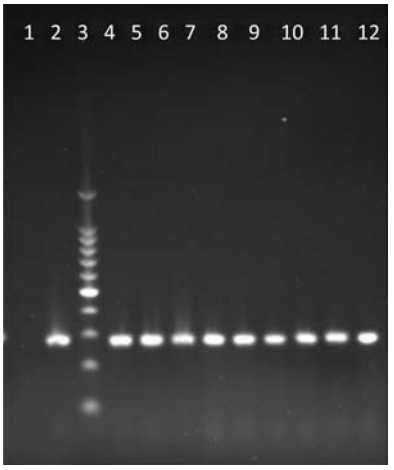

Fig. 3. Agarose electrophoresis of *nuc* gene specific PCR of *S. aureus*
Lane 1 – Negative control
Lane 2 - Positive control
Lane 3 – Ladder
Lane 4,5,6,7,8,9,10,11,12 – Positive samples (279 bp)

the identification of *S. aureus* organism isolated from bovine mastitis (Ciftci *et al*., 2009). *S. aureus* possess *nuc* gene which has species specific sequences which on amplification have the potential for detection as well as identification of *S. aureus* from infections (Brakstad *et al*., 1992; Costa *et al.,* 2004).

Panton Valentine Leucocidin is a phage encoded virulence factor which has major public health significance. Screening was performed for the presence of *PVL* in 18 isolates of *S. aureus* from bovine mastitis and none of them was found to have the presence of the gene. Similarly, absence of *PVL* was observed in other studies (Patel *et al*., 2021; Prashanth *et al*., 2011). Varying occurrence of *PVL* in bovine mastitis is observed throughout India, as high as 41.6 per cent (Mitra *et al*., 2013) and as low as 10.53 per cent (Shrivastava *et al*., 2018). Identification of *PVL* in bovine isolates is a rare finding and the frequency observed in other studies on bovine mastitis was attributed to the contamination of milk by milkermen carrying *PVL* containing *S. aureus* isolates (Fluit, 2011, Shrivastava *et al*., 2018). Unlike other leukotoxins, PVL is found to have weak action on bovine neutrophils, and this could be the probable reason for the absence of *PVL* in bovine strains and for its presence in human strains of *S. aureus* (Prevost *et al.,* 1995).

Conclusion

The present study concluded that there is presence of virulence gene in *S. aureus* associated with bovine mastitis isolated from Thrissur district. Further study of different virulence genes in a large study population must be conducted for extrapolating the data, to use it for epidemiological investigation and for the prevention and control of mastitis caused by *S. aureus.*

Conflict of interest

The authors declare that they have no conflict of interest.

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