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Molecular characterisation of virulence factors in *Staphylococcus aureus* associated with bovine subclinical mastitis

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Abstract
Subclinical mastitis is one of the alarming conditions affecting the quality of a dairy herd leading to a huge economic loss and cause decrease in the production and value of the milch animal. *Staphylococcus aureus* causes intramammary infection which leads chronic persistent infections. The organism produces virulence factors which aids in colonization of the mammary gland. The present study was performed for the detection of virulence factors in *S. aureus* associated with bovine subclinical mastitis. Screening of subclinical mastitis was performed in 100 lactating cows using California Mastitis Test, Electrical Conductivity and Somatic cell count. Using CMT, EC and SCC, 46 per cent, 38 per cent and 48 per cent of animals were found to be positive for subclinical mastitis, respectively. Milk samples from all quarters were subjected to isolation and identification of organisms and 40 per cent animals yielded positive results. From subclinical mastitis cases five isolates of *S. aureus* were obtained and confirmed by morphological, cultural, and biochemical characterisation. Selected virulence genes including *nuc*, *hla* and *PVL* were detected in the isolated *S. aureus* organisms. All the isolates were found to be positive for *nuc* gene, *hla* gene was detected in 20 per cent of isolates and no isolates possessed *PVL* gene. Varying frequencies of virulence factors were observed in this study. Understanding of the bovine clonal types prevalent in a herd will aid in the proper prevention and control of the mastitis.

Keywords: bovine, *S. aureus*, subclinical mastitis, virulence genes

Introduction
In India, the occurrence of subclinical mastitis is higher when compared to clinical mastitis, wherein a varying range of 10 per cent to 50 per cent of cattle population are affected (Lakshmi and Jayavardhan, 2016) [23]. The annual loss in case of mastitis in the US is approximately 2 billion dollars whereas in India it is 526 million dollars. More than 70 per cent of this loss is caused due to the presence of subclinical mastitis which farmers and dairymen are unaware (Varshney and Naresh, 2004) [39]. Subclinical mastitis is commonly identified using CMT, EC and SCC in a herd. *S. aureus* is the most commonly and frequently isolated pathogen in cases of intramammary infection in dairy animals (Gillespie *et al.* 2009) [16]. The successful infection caused by *S. aureus* is attributed to the different virulence factors which included a wide array of cell surface and secreted virulence factors. Expression of the virulence genes leads to adhesion of pathogen to host, defence against the immune system and destroy host cells. (Foster, 2005) [14]. Haemolysin α is one of the major virulence factors of *S. aureus* and that it produces cytotoxic effect on the host cells wherein they form heptameric pores on the cell membrane of target cell leading to extensive damage to cells by leakage of ATPs and critical ions. (Vandana *et al.*, 1997) [38]. *nuc* gene present in *S. aureus* encoded a thermostable extracellular nuclease which had been used for rapid, direct and early detection of the organism. (Sudhaharan *et al.*, 2015) [37]. *PVL* is a well-known toxin produced by *S. aureus* which is an important marker for virulent strains. It is reported that geographic variation persisted with respect to the prevalence of *PVL* producing strains and that the diffusion of this gene to varying MRSA lineages were mediated by bacteriophages (Shrivastava *et al*., 2018) [35]. The isolates carrying *PVL* gene identified in the study was linked to with contamination of milk by milkermen. Kot *et al.* (2016) [20] performed virulence gene profiling from samples collected from cows affected with subclinical mastitis and the presence of adhesion genes, proteases genes and superantigenic toxin genes were identified. The study also indicated the variation and diversity in the virulence genes containing isolates which caused mastitis. At present there are very few studies on the presence of virulence genes in case of *S aureus* associated with bovine subclinical mastitis and hence this study was performed.

Materials and Methods

Subclinical screening was performed in 100 lactating cows using CMT, EC and SCC. CMT was performed using the CMT reagent procured from Nice Chemicals Pvt. Ltd. Cochin, Kerala. After thorough cleaning of the udder and moping with a clean cloth, milk was collected from each quarter to the respective receptacle of the paddle and equal quantity of CMT reagent was squirted on to the paddle over the milk. In a horizontal plane, the paddle in to swirled gently in a rotating manner, and the reaction was scored within 15 seconds. For the detection of EC, a handheld electrical conductivity meter, Draminski Electronic Mastitis Detector, Poland was used, and instructions were followed as per manufacture guidelines were followed. Values were assigned to the milk samples according to the EC of the milk and any values below 250 were assigned as positive for subclinical mastitis. Milk collected from separate quarters were evaluated for SCC after staining by Modified Newmann stain. The milk smear was prepared by spreading the uniformly mixed sample of milk over one square centimeter area by keeping a template under the slide. The dried smears were stained by Modified Newman's stain (S024) by keeping the smear in the staining solution for 5 min as per product guidelines. The smears were gently washed in tap water, dried and examined under oil immersion objective of the microscope. Stained milk films were examined under oil immersion objective of the microscope and the number of somatic cells present in 25 fields were counted at random. The number of cells per field was arrived at by incorporating the dilution factor and microscopic factor. The SCC value of > 2,00,000 cells per ml of milk was taken as the criterion to declare the animal as subclinically infected.

Milk samples from each quarter was subjected for identification of organisms by morphological characterization, colony characteristics on selective media and biochemical reactions. (Barrow and Feltham, 1993 and Quinn *et al*., 2013) [7, 30]. Isolates presumptive of *S. aureus* were then further subjected for molecular characterization for confirmation.

Table 1: Details of primers used in PCR

Virulence genes	Genes	Primer sequence	Amplicon size (bp)	Reference
Alpha- Haemolysin	*hla*	F: GGT TTA GCC TGG CCT TC	534	Salasia *et al.* (2004) [33]
		R: CAT CAC GAA CTC GTT CG		
Thermonuclease	*nuc*	F: CCAAGCCTTGACGAACTAAAGC	279	Brakstad *et al.* (1992) [11]
		R: GCGATTGATGGTGATACGGTT		
Panton Valentine Leucocidin	*PVL*	F: CTGGACAAAACTTCTTGGAATAT	85	Pajic *et al.* (2014) [28]
		R:GATAGGACACCAATAAATTCTGGATTG		

The extraction of bacterial DNA was performed by snap chill method as described by (Vijayakumar and Jose, 2021) [40]. The presence of selected virulent factor genes, i.e., *nuc* for thermonuclease, *hla* for alpha - haemolysin and *PVL* for Panton Valentine Leucocidin were determined by polymerase chain reaction. The primers specific for them are 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 -20º C. The PCR were performed using the programmable S1000 Thermal cycler, BioRad, USA. PCR was performed by combining the reagents to a total volume of 25 µl reaction mixture. The PCR protocols for the selected virulence factor genes of *S. aureus* are shown in Table. 2. After completion of PCR reaction, the PCR products were subjected to submarine agarose gel electrophoresis.

Table 2: PCR protocol for amplification of virulence genes of *S. aureus*

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

Results and Discussion

The 100 animals were screened for subclinical mastitis by CMT, EC and SCC and the results are depicted in Table.3.

Table 3: Animal wise results for SCM

Result	Results for tests in percentage		
	CMT	EC	SCC
Positive	46	38	48
Negative	54	62	52

In this study, among the 100 animals screened, 46 per cent of the animals were found to be positive for SCM by CMT and similar high prevalence was reported by many researchers. Kader *et al.* (2002) [18] reported a prevalence of 46.6 per cent of SCM by CMT test and as high as 53.8 per cent by Krupa (2020) [21]. Lower occurrence of SCM by CMT was reported 39 per cent by Amritha Priya (2019) [5] and 27.2 per cent by Langer *et al.* (2014) [24]. The variation with respect to CMT scores can be attributed to the seasonal changes, geographical location, managemental practices and udder hygiene followed in the farm.

Shahid *et al.* (2011) [34] in their study found 65.2 per cent animals positive for SCM by EC using Draminski mastitis detector and reported that there was high sensitivity which was contradictory to the results from our study. Our results were in accordance with Langer *et al.* (2014) [24] and Raj (2017) [31] who found in their study a low prevalence of mastitis using Draminski mastitis detector. This signifies the fact that electrical conductivity should be used in conjunction with other screening tests for the detection of subclinical mastitis.

Electrical conductivity could be influenced by a lot of factors including stage of lactation and parity. It was also observed that EC increases with sample temperature, milk composition and milking time. (Nielen *et al.*, 1992) [27].

SCC is one of the reliable indicators for udder health and could be used for evaluating the effectiveness of preventive measures followed in a farm in conjunction with other diagnostic aids. In our study 48 per cent animals were found to have SCC greater than 2 lakhs per ml of milk and with a total of 105 quarters affected out of 383 quarters. This was similar to the findings by Amritha Priya (2019) [5] who found a 49.5 per cent of animals had a SCC above 2 lakhs per ml of milk. Badiuzzaman *et al.* (2015) [6] reported a SCC of 52 per cent and reported that SCC results exhibited results which were in accordance with the results of bacterial culture in their study. SCM screening performed based on SCC by Devi and Dutta (2018) [12] in Assam revealed a higher prevalence of SCM by SCC, which was as high as 93.3 per cent, cow wise and 90.26 per cent, quarter wise.

In the present study, overnight incubation of streaked milk samples collected from 100 animals revealed growth in case of 40 animals, therefore 40 per cent positivity of SCM was observed by bacteriological culture. Out of the 40 animals which yielded growth, 13 animals had growth in more than one quarter and 7 animals had more than one type of growth. Hence altogether, a total of 47 organisms were isolated for identification. Based on morphology, colony and biochemical characteristics the isolates were identified. (Table 4 and 5). The Gram-positive cocci isolated from subclinical mastitis in this study was *Staphylococcus aureus* (10.64 per cent), Coagulase Negative *Staphylococci* (48.94 per cent), *Micrococcus* sp. (17.02 per cent) and *Streptococcus* sp (4.25 per cent). The Gram-negative bacilli isolated from subclinical mastitis in this study includes *E. coli* (12.76 per cent) and *Klebsiella* sp. (6.39 per cent).

Table 4: Biochemical characterisation of Gram positive cocci from SCM

Sl. No	Catalase	Oxidase	Coagulase Test	IMViC	Urease test	Nitrate reduction test	Organism
1.	+	-	+	- + + +	+	+	*Staphylococcus aureus* (13.16 per cent)
2.	+	-	-	- + + +	+	+	Coagulase negative *Staphylococci* (60.53 per cent)
3.	+	+	-	- + - -	-	-	*Micrococcus* sp (21.05 per cent)
4.	-	-	-	- - - +	-	-	*Streptococcus* sp (5.26 per cent)

Table 5: Biochemical characterisation of Gram negative bacilli from SCM

Sl. No	Catalase	Oxidase	IMViC	Urease test	Nitrate reduction test	TSI test	Organism
1.	+	-	+ + - -	-	+	Y/Y/H_2S -ve	*E. coli* (66.67 per cent)
2.	+	-	- - + +	+	+	Y/Y/H_2S -ve	*Klebsiella* sp. (33.33 per ent)

This was in accordance with Ndahetuye *et al.* (2019) [26] who reported high prevalence of contagious pathogens isolated from subclinical mastitis with predominant proportion being CNS organisms (40. 2 per cent). This was contradictory to the findings by Alemu and Abraha (2017) [3] who reported *S. aureus* (33 per cent) as the dominant bacteria followed by Coagulase negative staphylococci (25 per cent). The high prevalence of contagious pathogens in herd could be attributed to unhygienic milking procedures, exposure of healthy cows to pathogens via milkermen or during machine milking from already affected cow and improper management measures. The results with respect to Gram negative bacilli were in contradictory with findings of Bhatt *et al.* (2012) [8], who reported *E. coli* as the most predominant organism from subclinical mastitis which was isolated from indigenous cattle breeds like Gir (30.97 per cent) and Kankrej (26.71 per cent). The high proportion of environmental pathogens like *E. coli* could be due to environmental contamination or due to the transient infection and not causing active infection. In the same study, evaluation in crossbred cattle was performed which yielded higher prevalence of *S. aureus* (48.08 per cent) as the major contagious pathogen responsible for sub clinical mastitis followed by *Klebsiella* sp (12.65 per cent) and the least prevalent was *E. coli* (5.77 per cent). This could be attributed to the fact that a specific group of organisms may be prevalent in a particular farm which may vary with geographical location that is responsible for the subclinical mastitis.

Virulence factors aid *S. aureus* in causing inflammation and infection by producing toxins and proteins which leads to the pathogenesis of disease (Bien *et* al., 2011) [9]. Virulence genes were identified by molecular characterization which yielded *nuc* gene in all isolates (100 per cent), *hla* in one isolate (20 per cent) and no isolate yielded results for *PVL* gene (0 per cent) in case of subclinical mastitis (Fig 1 and Fig 2.).

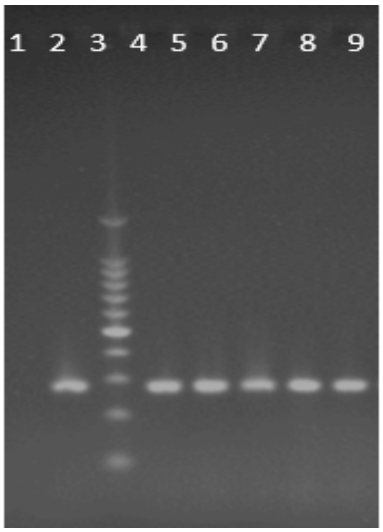

Fig 1: Agarose electrophoresis of *nuc* gene

Lane 1 – Negative control
Lane 2 - Positive control
Lane 3 – Ladder
Lane 4, 5, 6, 7, 8, 9– Positive samples (279 bp)

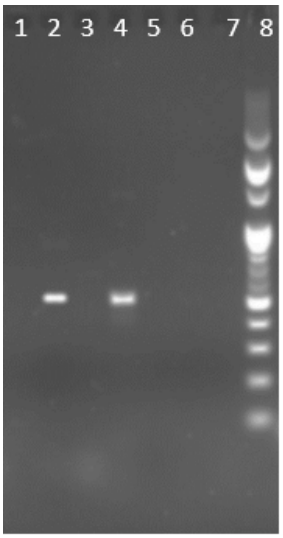

Fig 2: Agarose electrophoresis of *hla* gene

Lane 1 – Negative control
Lane 2 – Positive control
Lane 8 – Ladder
Lane 4 – Positive sample(534 bp)
Lane 3,5,6,7- Negative samples

The *nuc* gene is commonly used for species identification of *S. aureus*. With respect to *nuc* gene, similar reports to that of the present study was given by Kalorey *et al*. (2007) [19] who reported 36 out of 37 isolates of *S. aureus* yielded positive results for *nuc* gene and Salasia *et al*. (2004) [33] found all samples to be positive for *nuc* gene. However, in a study by Memon *et al.* (2013) [25] on selected virulence genes in Eastern China from bovine subclinical mastitis only 85 per cent of the isolates were found to possess *nuc* gene which was contradictory to our study.

Elsayed *et al*. (2015) [13] detected virulence factors from *S. aureus* both genotypically and phenotypically and found that there was a low prevalence of *hla* gene (34.4 per cent) which was in accordance with our study. Similar low prevalence of *hla* gene was observed by Ahmed *et al.* (2016) [1]. Only 18.8 per cent and 13 per cent of *S. aureus* possessed *hla* gene isolated from milk samples of subclinically affected cows and buffaloes respectively. This was contradictory to Xu *et al*. (2015) [41] who found a higher prevalence of *hla* gene in case of *S. aureus* strains isolated from bovine subclinical mastitis (96.4 per cent). Fursova *et al*. (2018) [15] performed study on exotoxin diversity studies in subclinical mastitis cases and found 70 per cent *S. aureus* isolates positive for *hla* gene.

Bonsaglia *et al.* (2018) [10] performed molecular epidemiological studies on *S. aureus* isolated from sub clinical mastitis and found an absence of *PVL* gene which was in accordance with our study. Silva *et al*. (2013) [36] and Aires-de-Sousa *et al*. (2007) [2] found similar absence of *PVL* gene in case of *S. aureus* from bovine milk samples. Low prevalence of *PVL* gene (3.4 per cent) was observed by Rossi *et al.* (2019) [32] while genotyping *S. aureus* strains from persistent subclinical mastitis cases. Kulangara *et al*. (2017) [22] found the presence of *PVL* gene in 5 isolates of *S. aureus* which was contradictory to our studies. Prevalence of *PVL* gene was observed by Algammal *et al.* (2020) [4] with 10 per cent of MRSA strains isolated from bovine subclinical mastitis positive for the gene. *PVL* positive samples are reported in many studies varying from 4.1 per cent to 96 per cent in various studies (Hoque *et al.*, 2018; Pumipuntu *et al*., 2019) [17, 29]. This could be associated with prevalence of MRSA strains with *PVL* gene and with transmission of gene through transduction to MSSA strains.

Conclusion

The virulence factors were screened in the *S. aureus* isolated from bovine subclinical mastitis. In five isolates, out of the three virulence genes, two virulence genes were detected. The selected virulence factors were found in varying frequencies with one isolate had both *nuc* and *hla* gene, and four isolates only had *nuc* gene. No isolates had presence of *PVL* gene. The variation in the presence of different virulence genes in case of bovine mastitis could be attributed to the fact that there is the presence diverse strains of *S. aureus* and the pathogenicity caused by each strain varies depending on the virulence genes they possess and the virulence factors they produce.

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