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Detection of Virulence Genes in Methicillin Resistant *Staphylococcus aureus* from Bovine Subclinical Mastitic Milk

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ABSTRACT

Subclinical mastitis is a crucial problem influencing dairy animals across the globe. *Staphylococcus aureus* is generally considered to be a contagious udder pathogen, which mainly spreads within and between cows at milking. It can also be transmitted through the consumption of raw milk and milk products. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major concern for human health which can cause severe infectious diseases in humans. In the present study, bovine subclinical mastitic milk samples were processed for the isolation, identification and molecular characterization of the methicillin resistant *Staphylococcus aureus*. In total, 400 quarter milk samples were collected from 10 organized dairy farms of Udaipur district, Rajasthan from June 2021 to October 2021. These samples were screened for subclinical mastitis through Modified California Mastitis Test (MCMT) and Somatic Cell Count (SCC). Out of the 85 *S. aureus* isolates obtained from bovine subclinical mastitic milk (n=133), 5 isolates were confirmed as MRSA on the basis of phenotypic antibiotic susceptibility pattern giving a prevalence rate of 5.89%. All the MRSA isolates (n=5) were found to be positive for species specific (23S *rRNA*) and thermonuclease gene (*nuc*). Prevalence of virulence genes *viz*., *clfA*, *fnbA* and *hlb* in MRSA isolates was 60%, 80% and 40%, respectively. The presence of these virulent MRSA strains in milk indicates a potential public health risk.

HIGHLIGHTS

- The virulence potential of MRSA isolated from bovine subclinical mastitic milk samples.
- The *nuc, clfA, fnbA* and *hlb* genes were prevalent in the MRSA isolates.

Keywords: Mastitis, MRSA, molecular characterization, public health

Bovine mastitis is one of the most costly disease of the dairy sector (Schmidt *et al*., 2015). It is a multi-etiological and complex disease, which is characterized by the inflammation of udder parenchyma (Gomes *et al*., 2016; Sharma *et al*., 2008) and is caused by a wide range of pathogens. Animals with subclinical mastitis (SCM) do not exhibit gross abnormalities in milk or udder but the milk production decreases with an increase in somatic cell count (Khan and Khan, 2006) as a result of the host immune responses. *Staphylococcus aureus* is a potential pathogen that is responsible for intramammary infections which decreases the health status of cows, leading to huge economic losses (Wang *et al*., 2016). *S. aureus* is generally considered to be a contagious udder pathogen, which mainly spreads within the dairy ecosystem. The pathogenicity of *S. aureus* is associated with the presence of a wide range of virulence factors enabling adherence, colonization, invasion of the mammary cells, evasion of the immune defense mechanism and survival in the host environment (Memon *et al.,* 2013). These virulence factors

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are highly stable to heat or proteolytic enzymes which help the bacteria to survive and multiply in the mammary gland (De Freitas Guimaraes *et al*., 2013).

Of the wide variety of exoproteins produced by *S. aureus*, α and β- hemolysins are one of the important factors in the pathogenesis that cause disease through haemolysis of erythrocytes. Beta haemolysin contains sphingomyelinase that is more active against sheep and bovine erythrocytes (da Silva *et al.,* 2005) and is governed by *hlb* gene. The β-toxin is a Mg^{+2}-dependent sphingomyelinase C, which degrades sphingomyelin in the outer phospholipid layer of the membrane (Linehan *et al*., 2003).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major concern for human health which can cause severe infectious diseases in humans including; pyogenic endocarditis, suppurative pneumonia, otitis media, osteomyelitis, pyogenic infections of the skin, soft tissues, and septic arthritis (Gajdacs and Urban, 2019). The gene responsible for methicillin resistance that is *mecA*, is part of a mobile genetic element found in MRSA strain which can be detected by PCR. Thus, the present study was designed to detect the occurrence of *nuc*, *clfA*, *fnbA* and *hlb* genes in methicillin resistant *S. aureus* isolates obtained from bovine subclinical mastitic milk.

MATERIALS AND METHODS

Sample collection

A total of 400 bovine quarter milk samples were collected from 10 organized dairy farms (10 animals from each dairy/40 quarter milk samples from each dairy) of Udaipur district (Rajasthan). Milk samples were collected following standard aseptic procedures. The udder was washed with clean water and wiped with clean and dry cloth. Teats of the animal and hands of the milker were disinfected with alcohol. Milk sample (40 ml) was collected in sterile tubes after discarding the first few strips of milk. The samples were transported to the laboratory within 2 hours in chilled condition by using ice packs. The sampling of milk was done from June 2021 to October 2021.

Determination of subclinical mastitis

The modified California mastitis test (MCMT) was performed and interpreted as described by (Kandeel *et al*., 2018).

Quarter milk sample (2 ml) was placed in the appropriate chamber of the CMT plastic paddle and mixed with 2 ml of modified CMT reagent at ambient temperature by gently moving the paddle in a circular motion in horizontal plane. The positive CMT reaction was reflected by the degree of precipitation or gel formation that occured. Somatic cell count (SCC) was measured by Lactoscan somatic cell counter (Insif, India). Milk sample (100 µl) was mixed with the reagent, containing the fluorescent dye (Sofia Green). Only 8 µl of the dyed sample was pipetted on the measuring chamber of disposable lactochip. Lactoscan SCC system measured the dyed cells by high-end direct fluorescent cell counting software. The SCC value exceeding 2,00,000 cells/ml of milk was taken as the criteria to declare the animal as subclinically infected, if no gross abnormality was noticed in the milk or udder (Smith *et al*., 2001).

Isolation and identification of methicillin resistant *S. aureus*

The isolation and identification of the organism was done on the basis of cultural, morphological and biochemical characteristics as per the method described by Cowan and Steel, 1974; Quinn *et al*., 1994; Bauer *et al.*, 1966.

Molecular characterization of MRSA

Isolation of DNA from pure culture was undertaken by using the Nucleo-pore gDNA fungal/bacterial mini kit. The genotypic confirmation of *S. aureus* on the basis of 23S *rRNA* gene was carried out as per method described by Straub *et al*., 1999. Similarly, PCR for detection of

nuc, *clfA*, *fnbA* and *hlb* genes was carried out as per the method described by Barski *et al*., 1996; Stephan *et al*., 2001; Nashev *et al*., 2004; Booth *et al*., 2001, respectively. The primer pairs used in PCR are mentioned in Table 1. The reaction mixture was optimized to contain 12.5 µl Green taq PCR master mix, 10 nmol (0.5 µl) of each forward and reverse primer, 10.5 µl nuclease free water and 1µl of DNA template. The reaction was performed in the thermal cycler with pre-heated lid (lid temp. =105°C). The cycling conditions are mentioned in Table 2. The PCR products were analyzed by electrophoresis on 1% agarose gel at 70V for 60 minutes.

Table 1: Primers used for the molecular characterization of *S. aureus*

Sl. No.	Gene	Primer sequence (5´ to 3´)	Size (bp)	Reference
1	23S *rRNA*	**F: -**ACGGAGTTACAAAGGACGAC	1250	(Straub *et al*., 1999)
		R: -AGCTCAGCCTTAACGAGTAC		
2	*nuc*	**F: -**GCGATTGATGGTGATACGGTT	279	(Barski *et al*., 1996)
		R: -AGCCAAGCCTTGACGAACTAAAGC		
3	*clfA*	**F: -**GGCTTCAGTGCTTGTAGG	1000	(Stephan *et al*., 2001)
		R: -TTTTCAGGGTCAATATAAGC		
4	*fnbA*	**F: -**GCGGAGATCAAAGACAA	1279	(Nashev *et al*., 2004)
		R: -CCATCTATAGCTGTGTGG		
5	*hlb*	**F: -**GCCAAAGCCGAATCTAAG	833	(Booth *et al*., 2001)
		R: -CGCATATACATCCCATGGC		

Table 2: Steps and conditions of thermal cycling for different primer pairs in PCR

Primers	Cycling conditions				
	Initial denaturation	**Denaturation**	**Annealing**	**Extension**	**Final extension**
23S *rRNA*	94ºC	94ºC	54ºC	72ºC	72ºC 5 minutes
	5 minutes	40 second	1 minute	1 minute	
	Repeated for 30 cycles				
Nuc	94ºC	94ºC	58ºC	72ºC	
	5 minutes	1 minute	1 minute	1 minute	
	Repeated for 30 cycles				
clfA	94ºC	94ºC	57ºC	72ºC	
	5 minutes	1 minute	1 minute	1 minute	
	Repeated for 35 cycles				
fnbA	94ºC	94ºC	50ºC	72ºC	
	5 minutes	1 minute	30 seconds	1 minute	
	Repeated for 30 cycles				
hlb	94ºC	94ºC	50ºC	72ºC	
	5 minutes	1 minute	1minute	1 minute	
	Repeated for 30 cycles				

RESULTS AND DISCUSSION

Prevalence of MRSA in bovine subclinical mastitic milk

In the present study, out of 400 quarter milk samples analyzed, MCMT was found to be positive in 37% (n= 148/400) of the samples and 133 quarter milk samples had somatic cells exceeding 2 lakh cells/ml. From a total of 133 subclinical mastitic milk samples, 85 isolates were confirmed as *S. aureus* on the basis of morphological, cultural and biochemical characteristics. Among them, 5 *S. aureus* isolates were confirmed as MRSA on the basis of phenotypic antimicrobial susceptibility test giving a prevalence of 5.89%. Similar finding was reported by Aslantas and Demir, 2016 who obtained 5 MRSA out of 112 *S. aureus* isolates showing the prevalence as 4.5%. While, higher prevalence of MRSA was reported by

Kumar *et al*., 2010; Yang *et al*., 2020 as 7.2% and 14.65% among the *S. aureus* isolates recovered from subclinical mastitis, respectively.

Prevalence of virulence genes in MRSA

The genotypic confirmation was carried out with a PCR based method involving specific primer targeted against 23S *rRNA* gene yielding an amplicon of 1250 bp in all the 5 MRSA isolates (Fig. 1).

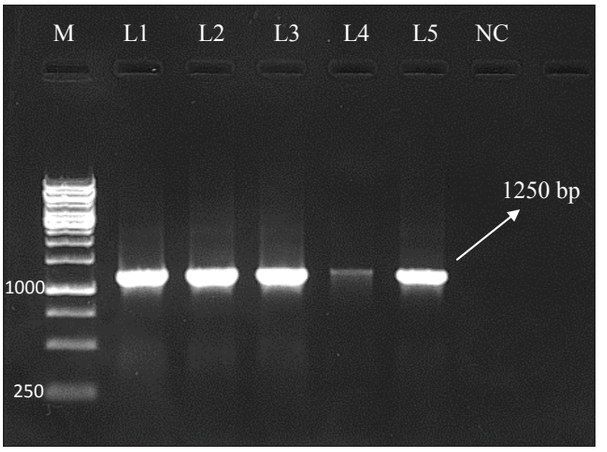

Fig. 1: Agarose gel showing PCR amplified product (1250 bp) for 23S *rRNA* gene in MRSA isolates from bovine subclinical mastitic milk; M= 1kb DNA ladder, positive samples (L1=M1, L2=M2, L3=M3, L4=M4, L5=M5, NC= negative control)

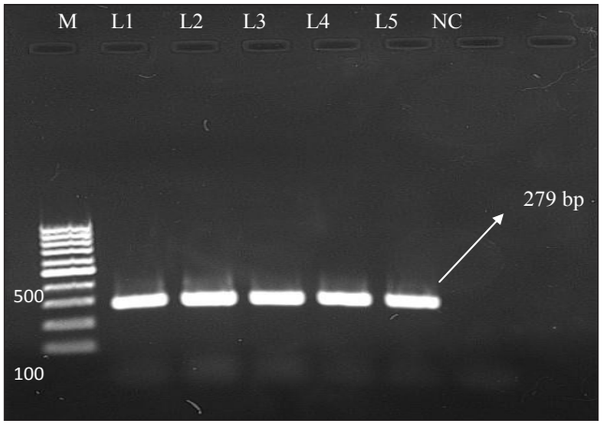

Fig. 2: Agarose gel showing PCR amplified product (279 bp) for *nuc* gene in MRSA isolates isolated from bovine subclinical mastitic milk; M= Ladder (100 bp), positive samples (L1=M1, L2=M2, L3=M3, L4=M4, L5=M5, NC= negative control)

For virulence gene detection in *S. aureus* isolates, *nuc* gene coding for the extracellular thermostable nuclease protein was targeted in PCR, which was found to be prevalent in all the MRSA isolates (Fig. 2). Kumar *et al*., 2011; Bhati *et al*., 2018 also reported 100% prevalence of *nuc* gene among *S. aureus* isolates. The detection of *clfA* gene revealed its presence in 3 MRSA isolates (Fig. 3). Similar findings were reported by Momtaz *et al*., 2010; Memon *et al*., 2013; Aslantas and Demir, 2016 in which they found 73.25%, 59% and 66.96% of the *S. aureus* isolates as possessing the *clfA* gene, respectively. The clumping factor A participates in the infection process by facilitating bacterial binding leading to enhancement of its virulence (Karahan *et al*., 2011).

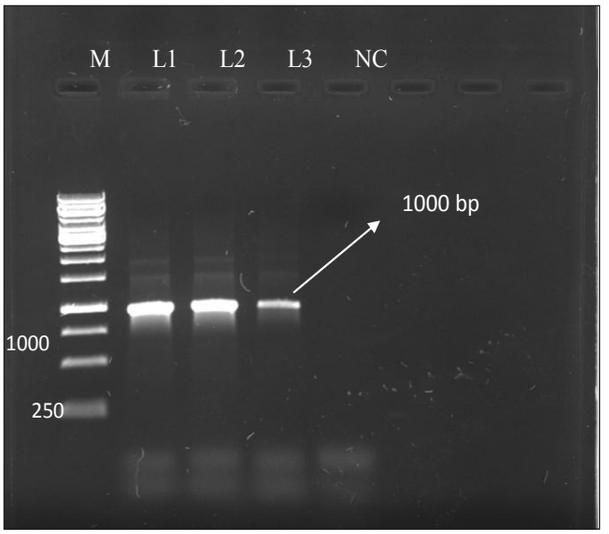

Fig. 3: Agarose gel showing PCR amplified product (1000 bp) for *clfA* gene in MRSA isolates from bovine subclinical mastitic milk. M= 1kb DNA ladder, positive samples (L1=M3, L2=M4, L3=M5, NC= negative control)

Similarly, the *fnbA* gene was found in 4 MRSA isolates (Fig. 4) which was in accordance with the reports of Aslantas and Demir, 2016 who found prevalence of the *fnbA* gene as 77.67%. The detection of *hlb* gene revealed its presence in 2 MRSA isolates giving a prevalence of 40% (Fig. 5) which was similar to the findings of Elsayed *et al*., 2015 who found prevalence of the *hlb* gene as 37.5%. In other reports higher prevalence was revealed by Yadav *et al*., 2015; Pumipuntu *et al*., 2019 as 84.3% and 70.83%, respectively.

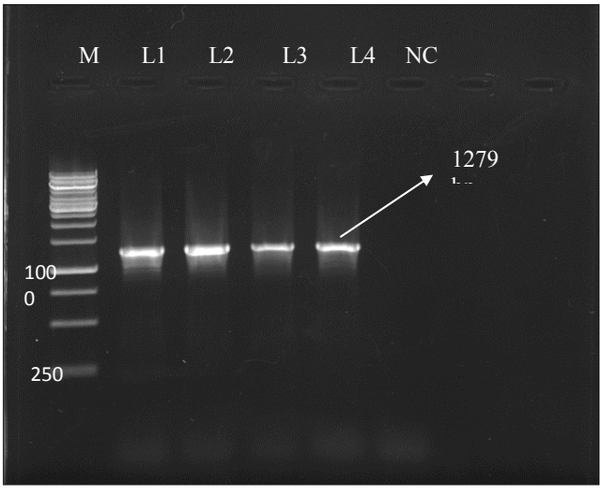

Fig. 4: Agarose gel showing PCR amplified product (1279 bp) for *fnbA* gene in MRSA isolates from bovine subclinical mastitic milk; M= 1kb DNA ladder, positive samples (L1=M2, L2=M3, L3=M4, L4=M5, NC= negative control)

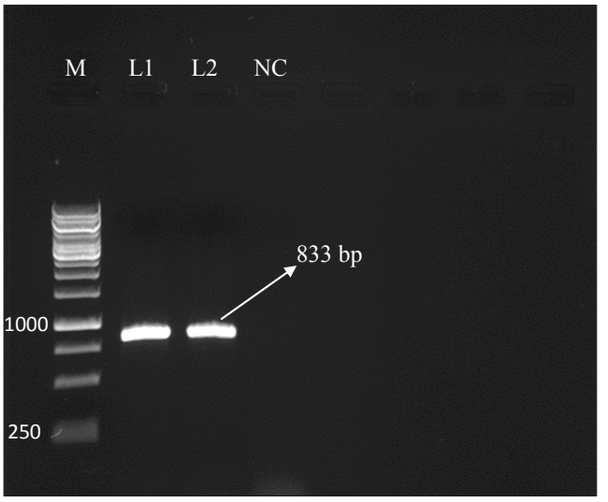

Fig. 5: Agarose gel showing PCR amplified product (833 bp) for *hlb* gene in MRSA isolates from bovine subclinical mastitic milk. M= 1kb DNA ladder, positive samples (L1=M3, L2=M4, NC= negative control)

In the present study, the PCR assays were used for the detection and characterization of virulence factors of *S. aureus*. Huma *et al.* (2022) have also used the *S. aureus* toxic genes for molecular characterization of bacteria. Further studies are required for the genomic assessment of antibiotic resistant strains possessing virulence factors which can help in the development of suitable disease control strategies. Moreover, the isolation of MRSA from bovine subclinical mastitic milk samples indicates towards the public health risk due to the consumption of raw milk and milk products. Therefore, implementation of hygienic milk production practices with strict compliance of mastitis preventive strategies should be emphasized.

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