International Journal of Livestock Research, Vol. 10 (12) Dec' 2020
http://www.ijlr.org
eISSN: 2277-1964
NAAS Rating 2020 – 5.36

Original Research

Molecular Detection of Virulence Genes of *Staphylococcus aureus* Isolated from Bovine Mastitis

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How to cite this paper: Chaudhari, D., Bhagat, A., Patel, J., Patel, Y., Adroja, A., Vyas, T., & Parmar, A. (2020). Molecular Detection of Virulence Genes of Staphylococcus aureus Isolated from Bovine Mastitis. *International Journal of Livestock Research*, 10(12), 143-154. doi: http://dx.doi.org/10.5455/ijlr.20200817041535

Received : Aug 13, 2020
Accepted : Oct 23, 2020
Published : Dec 31, 2020

Abstract

Staphylococcus aureus is often responsible for a number of diseases in humans and animals due to the production of different virulence factors. Therefore, present study investigated the virulence genes of Staphylococcus aureus isolated from bovine mastitis. Out of 52 isolates of Staphylococcus aureus, all the isolates were found negative for icaA gene whereas 18 (34.61 %) isolates were found positive for icaD gene producing 381 bp amplicon. In detection of hemolysine gene hlA and hlB, out of 52 isolates of Staphylococcus aureus, 15 (28.84 %) and 11 (21.15 %) isolates were found positive for presence of hlA and hlB gene, respectively. Out of 52 isolates of Staphylococcus aureus, 43 (82.69 %) isolates were found positive for coa gene in which single amplicons of 550, 680 and 850 bp were detected among 19 (36.53 %), 11 (21.15 %) and 13 (25.00%) isolates, respectively. In the detection of X-region of Protein A in spa gene by PCR, 18 (34.61 %) isolates isolates of Staphylococcus aureus out of 52 were found positive in which single amplicons of 180, 206 and 270 bp were detected among 6 (11.53%), 4 (07.69%) and 8 (15.38%) isolates, respectively indicative of polymorphisms of spa gene. These results revealed that mastitis associated Staphylococcus aureus among bovines is able to accumulate different virulence factors making the treatment of infections difficult.

Keywords: *Staphylococcus aureus*, Virulence Genes, Mastitis and PCR

Introduction

Mastitis is an inflammation of mammary gland and characterized by physical, chemical, and bacteriological changes in milk and pathologic changes in the glandular tissue (Lakshmi *et al.,* 2016). Mastitis can be defined as clinical (grossly evident changes as to milk, the gland or the whole animal.) or as subclinical (Duguma *et al*., 2014). *Staphylococcus aureus* is the most prevalent in intramammary infections and is responsible for more than 80.00 per cent of the cases (Pellegrino *et al.,* 2011 and Song *et al.,* 2016).

Pathogenicity of staphylococcus associated with mastitis is an extremely important feature in the disease process that requires a better understanding. Ability of *Staphylococcus aureus* to cause various infections and intoxication results from the production of different virulence factors (Aung *et al.,* 2011). Slime production is considered as a virulence factor that inhibits the immune response of the host and facilitates the adhesion of the pathogen (Atkin *et al.,* 2014). The main component of the *Staphylococcus aureus* slime is the exopolysaccharide poly-N-acetyl-β-1,6-glucosamine (PNAG), synthesized by enzymes encoded in the intercellular adhesion (*ica*) locus. The *ica* locus belongs to the "accessory genes" of the genome and contains *ica*A, *ica*B, *ica*C and *ica*D genes of which *ica*A and *ica*D play a significant role (Arciola *et al.,* 2001). The *ica*A gene encodes enzyme N-acetylglucosaminyl transferase whose enzymic activity becomes significant only in the case of *ica*D gene expression (Arciola *et al.,* 2015).

In addition, *Staphylococcus aureus* produces a wide array of virulence elements including, enzymes and toxins (Da Silva *et al.,* 2005). Hemolysins are considered to be the most important virulent elements in the development of the disease (Ariyanti *et al.,* 2011). The α and β hemolysins are the most important virulent factors in the development of the disease. They are pore-forming exotoxins that induce proinflammatory changes in mammalian cells, inactivate the immune system by their direct cytotoxic effect, and degrade tissues, providing bacteria with nutrients and facilitating spreading to new sites. The α and β hemolysin are encoded by *hl*A and *hl*B gene, respectively. Both *hl*A and *hl*B genes are controlled by gene regulatory accessory *agr* (Bownik and Swicki, 2008).

Coagulase is a major virulent factor, which is an enzyme secreted by *Staphylococcus aureus* causing the clotting of plasma in the host. It causes conversion of fibrinogen to fibrin and fibrin production may shield staphylococcus from phagocytosis. Clinical microbiologists consider coagulase production by *Staphylococcus aureus* as an important criterion for identification of *Staphylococcus aureus.* Although the coagulase tube test is the standard phenotypic routine test used to identify *Staphylococcus aureus* in biological samples, several groups have implemented the molecular analysis of the coagulase as an accurate defined test. The *coa* gene encoding coagulase protein is highly polymorphic because of the variable sequences (81 bp tandem repeats) at its 3' end which allows differentiation of *Staphylococcus aureus* species (Goh *et al.,* 1992). This *coa* gene polymorphism is utilized as an epidemiological marker and typing is performed with primers homologous to a conserved region within the *coa* gene (Schwarzkoph *et al.,* 1995). Since the number of repetitive sequences varies within the *coa* gene, the resulting polymerase chain reaction (PCR) products of individual strains can be of different lengths.

Protein A is a virulence factor with molecular weight of 42KD (Palmqvist *et al.,* 2002). It is covalently anchored to the peptidoglycan of *Staphylococcus aureus.* Protein A of *Staphylococcus aureus* encoded by *spa* gene is considered as one of the important virulent factors in the development and severity of mastitis (Sharma *et al.,* 2000 and Akineden *et al.,* 2001). The gene encoding protein A (*spa*) is composed of some functionally distinct regions: IgG Fc binding region (*spa*-IgG) and X-region (*spa*-X) at C terminus, a sequence required for cell wall attachment. The repetitive part of region X consists of up to 12 units each with a length of 24 nucleotides is highly polymorphic with respect to the number and sequence of repeats. Diversity of X region causes variation in Protein A of *Staphylococcus aureus* (Wichelhaus *et al.,* 2001 and Mitani *et al.,* 2005). Strains with more than seven repeats in the X region tended to be epidemic and with seven or fewer repeat units tended to be non-epidemic isolate (Frenay *et al*., 1994).

This study aimed to detect virulence genes of *Staphylococcus aureus* isolated from bovine mastitis.

Materials and Methods

Isolation and Identification of *S. aureus*

All the 275 bovine mastitic milk samples were inoculated on the Nutrient Agar (NA) plates for primary isolation. Thereafter, colonies showing golden yellow pigmented or white colony colour indicative of presumptive

Staphylococcus aureus were transferred to Mannitol Salt Agar (MSA) plates which is considered as selective medium for Genus Staphylococcus. The mannitol fermenting colonies of presumptive *Staphylococcus aureus* were transferred to nutrient agar slants for further identification. All the probable *Staphylococcus aureus* isolates were subjected to molecular identification using species-specific *thermonuclease* (*nuc)* gene.

Extraction of Bacterial DNA

Genomic DNA was extracted from freshly grown culture as per the method described by Younis *et al.* (2018). In brief, 3 to 5 bacterial colonies were picked up and suspended in 50 µl deionized water followed by boiling for 5 minutes and centrifuging at 10000 rpm for 1 min. The supernatant was then transferred and used as the DNA template for further molecular characterization**.**

Molecular Detection of Virulence Genes

Detection of virulence genes encoding *ica*A and *ica*D implicated in the production of slime; *hl*A and *hl*B implicated for hemolysin production; *coa* gene that codes for coagulase enzyme and *spa* gene that codes for protein was carried out using PCR. Details of oligonucleotide primers used in the present study are mentioned in Table 1. The PCR amplification for virulence gene *viz*. icaA, *ica*D, *hl*A, *hl*B, *spa* and *coa* gene was performed in 20 µl reaction volume in 0.2 ml thin-walled PCR tubes using a Veriti 96-Well Thermal Cycler (Applied Biosystems). Reaction mixture for detection of virulence gene *viz. ica*A, *ica*D, *hl*A, *hl*B, *spa* gene comprised of 10.0 µl of 2X PCR Master Mix, 3.0 µl of Nuclease Free Water, 5.0 µl of DNA Template, and 1.0 (10 pmole/µl) of each gene-specific Forward/ Reverse primers whereas Reaction mixture for detection of *coa* gene contained 10.0 µl of 2X PCR Master Mix, 2.0 µl of Nuclease Free Water, 5.0 µl of DNA Template, and 1.5 (10 pmole/µl) of each Forward and Reverse primers. The cyclic conditions for each reaction are given in Table 2.

Table 1: Details of Primers used for amplification of virulence genes of *Staphylococcus aureus*

Gene designated	Primer sequence (5'- 3')		Size of amplifid products (bp)	References
*ica*A	Forward	CCTAACTAACGAAAGGTAG	1315 bp	Vasudevan *et al.*, 2003
	Reverse	AAGATATAGCGATAAGTGC		
*ica*D	Forward	AAACGTAAGAGAGGTGG	381 bp	Vasudevan *et al.*, 2003
	Reverse	GGCAATATGATCAAGATAC		
*hl*A	Forward	CTGATTACTATCCAAGAAATTCGATTG	209 bp	Jarraud et al., 2002
	Reverse	CTTTCCAGCCTACTTTTTTATCAGT		
*hl*B	Forward	GTGCACTTACTGACAATAGTGC	309 bp	Jarraud et al., 2002
	Reverse	GTTGATGAGTAGCTACCTTCAGT		
coa	Forward	ATAGAGATGCTGGTACAGG	Variable	Hookey *et al*. (1998)
	Reverse	GCTTCCGATTGTTCGATGC		
spa	Forward	CAAGCACCAAAAGAGGAA	Variable	Frenay *et al.* (1996)
	Reverse	CACCAGGTTTAACGACAT		

Table 2: Cyclic conditions for PCR amplification of *Staphylococcus aureus* virulent genes

Gene	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles
*ica*A	92ºC/5 min.	92ºC/ 1 min.	49 ºC/ 50 sec.	72ºC/1 min.	72ºC/7 min.	**30**
*ica*D	92ºC/5 min.	92ºC/ 1 min.	49 ºC/ 50 sec.	72ºC/1 min.	72ºC/7 min.	**30**
*hl*A	95ºC/5 min.	94ºC/60 sec.	55 ºC/30 sec.	72ºC/1 min.	72ºC/10 min.	**30**
*hl*B	95ºC/5 min.	94ºC/60 sec.	55 ºC/30 sec.	72ºC/1 min.	72ºC/10 min.	**30**
coa	94ºC/5 min.	94ºC/30 sec.	60 ºC/45 sec.	72ºC/2 min.	72ºC/7 min.	**30**
spa	94ºC /5 min.	95ºC/1 min.	55 ºC/1 min.	72ºC/1 min.	72ºC/5 min.	**30**

Electrophoresis and Gel Documentation

Agarose gel (1% w/v) was made by heating the appropriate amount of agarose with 30 ml 1X Tris-acetate EDTA (TAE) buffer in a 500 ml Erlenmeyer flask. The flask was cooled to 60°C, and ethidium bromide added to the final concentration of 0.5 µg/ml. The warm agarose solution was poured into a plastic holder with suitable comb and allowed to completely set at room temperature for 30 min. Mixture of PCR product (5 µl) and 6X gel loading dye (2 µl) was loaded in separate wells on the submerged gel. Standard molecular weight marker, 100 bp DNA Ladder (Gelpilot), was also loaded in one well. The voltage 80 V was applied across the gel until tracking dye (Bromophenol blue) migrated to appropriate distance. The gel was removed and DNA bands were visualised under ultraviolet illumination and photographed with Gel Documentation System (Invitrogen life technology E-Gel imaginig system, Israel). The molecular sizes of the DNA bands were analyzed in relation to molecular weight DNA ladder.

Results and Discussion

In the present study, 52 (85.24) isolates were detected from 275 samples collected which revealed colonies typical for *S. aureus* which were later confirmed by PCR amplification of the *nuc* gene which produced an amplicon of 270 bp. Worldwide, mastitis has been reported to be one of the most common infectious diseases affecting dairy Cattle and is the most economically affecting disease of dairy industry. *Staphylococcus aureus* is considered to be the most common mammary pathogen found in bovine mastitis in the whole world and is an obvious contributor to milk contamination (Andre *et al.,* 2008). Epidemiologic studies indicate that *Staphylococcus aureus* produces a large array of virulence factors and it is believed that there is a relationship between the severity of mastitis and the virulence factors produced by *Staphylococcus aureus* (Akineden *et al.,* 2001). Virulence factors of *Staphylococcus aureus* allow the bacteria to attach, colonize and invade the host (Soares *et al.,* 2017). Hence, the presence of virulence genes was investigated in *Staphylococcus aureus* isolated from bovine mastitis in this study.

***ica*A and *ica*D genotyping**

In the present study, all the 52 isolates of *Staphylococcus aureus* isolates were found negative for *ica*A gene whereas 18 (34.61%) isolates were detected positive for *ica*D gene producing 381 bp amplicon. (Fig. 1) Similarly, De Aimeida *et al*. (2017) reported none of the 32 *Staphylococcus aureus* strains isolated from buffalo milk, milking machines and milker's hands positive for *ica*A; only seven were positive for *ica*D gene. The findings of Ciftci *et al.* (2009) speak in favor of results from this study, as they also determined only the presence of *ica*D gene in a portion of *Staphylococcus aureus* mastitic isolates. Szweda *et al.* (2012) pointed out that some mutations in *ica* genes are possible and this may be the reason for the failed amplification of *ica*A genes.

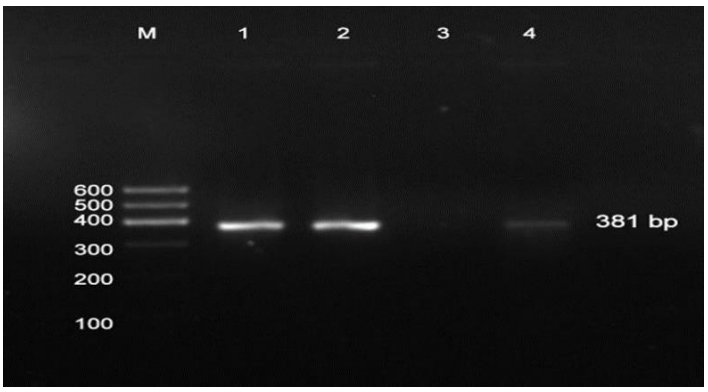

Figure 1 : PCR amplification of icaD gene of Staphylococcus aureus isolates
Lane M : 100 bp DNA marker; Lane 1, 2 & 4 : Positive sample (381 bp amplicon); Lane 3 : Negative sample

In contrast to the present findings, Goyal *et al*. (2014) reported that 65.00% (26/40) isolates obtained from cattle mastitis harboured *ica*A gene, but all of the isolates are found negative for *ica*D gene. Dhanawade *et al.* (2010) examined that among the group of 102 *Staphylococcus aureus* mastitic isolates from India, only 36 revealed the presence of both the genes. On the other hand, several authors showed presence of the *ica* locus genes in all *Staphylococcus aureus* clinical isolates analyzed in their studies (Knobloch *et al.,* 2002, Atsthan and Shamsudin, 2011 and Szweda *et al.,* 2012). These variations could be due to circulations of different clones of *Staphylococcus aureus* in different regions. The presence of the *ica*D gene alone might indicate the existence of the *ica* locus in a

total of 34.61% investigated *Staphylococcus aureus* mastitic isolates. Results of the present study indicate the existence of potential biofilm producer strains in bovine mastitis.

The first step in the pathogenesis of mastitis caused by *Staphylococcus aureus* is the adherence and production of slime (biofilm) which enables adhesion of bacteria to the epithelium of mammary glands and also increases antibiotic resistance. It also facilitates persistence of microorganisms in the host tissue by protecting the bacterial cells against the mechanisms of the host defense (Melchior *et al.*, 2007). The most studied biofilm mechanism in *Staphylococcus aureus* is *ica* operon (*ica*A and *ica*D genes) dependent mechanism and previous research demonstrated that the majority of clinical isolates, both from human and bovine mastitis contain the *ica* operon (O'Gara *et al.*, 2007). Therefore, PCR assay standardized for detection of the *ica*A and *ica*D genes can determine the potential of *Staphylococcus aureus* isolates to produce biofilm and may help in elimination of such slime forming bacteria to control mastitis (Bhati *et al.*, 2018).

*hl*A and *hl*B genotyping

In the present study, out of 52 isolates subjected to *hl*A gene amplification, 15 (28.84%) isolates produced 209 bp amplicon (Fig. 2). which corroborates the findings of Ali *et al.* (2018) and El sayed *et al.* (2015) who observed 33.33 and 34.40 per cent *Staphylococcus aureus* isolates respectively from bovine clinical mastitis carried *hl*A gene. In contrast to the present findings, Salasia *et al.* (2011), Yang *et al.* (2012) and Wang *et al.* (2016) observed high frequency of (100.00%, 85.00% and 94.30%) *hl*A gene in *Staphylococcus aureus* isolated from clinical mastitis, respectively. The α-haemolysin, encoded by *hl*A gene, has been suggested to be involved in peracute, gangrenous bovine mastitis.

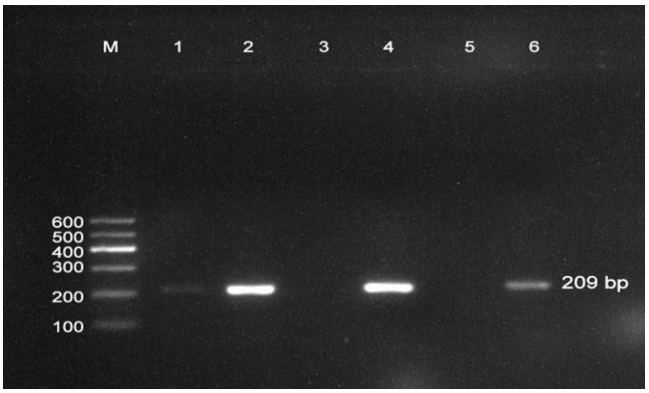

Figure 2: PCR amplification of hlA gene of *Staphylococcus aureus* isolates
Lane M: 100 bp DNA marker; Lane 1, 2, 4 & 6: Positive sample (209 bp amplicon); Lane 3 & 5: Negative sample

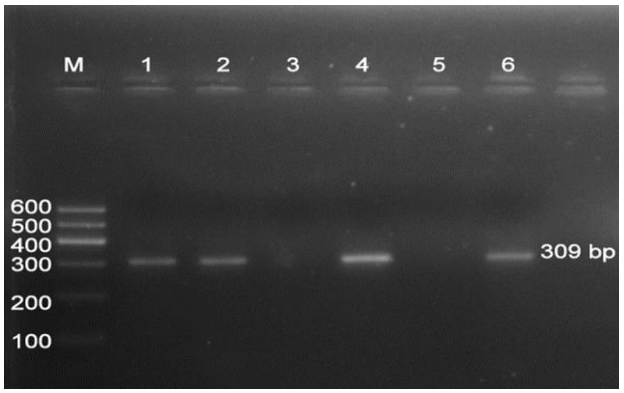

Figure 3: PCR amplification of hlB gene of *Staphylococcus aureus* isolates
Lane M: 100 bp DNA marker
Lane 1, 2, 4 & 6: Positive sample (309 bp amplicon)
Lane 3 & 5: Negative sample

With regard to *hl*B gene, it was detected in 11 out of 52 *Staphylococcus aureus* isolates with an incidence of 21.15 per cent (Fig. 3). The present results correspond significantly with similar results obtained by Ali *et al.* (2018) who reported 16.66% incidence of *hl*B gene in *Staphylococcus aureus.* In other studies, higher percentages of 97.00, 84.00, 71.00 and 79.10 per cent *hl*B gene in *Staphylococcus aureus* isolates were reported by Larsen *et al.* (2002), Salasia *et al.* (2011), Memon *et al.* (2013) and Wang *et al.* (2016), respectively. These different frequencies of *hl*A and *hl*B gene in the present and previous studies might be due to the different animal populations studied or the implemented methodologies, among other factors.

Hemolysins are considered the most important cytolytic exotoxins produced by *Staphylococcus aureus* which affecting the cell membrane. Alpha (α-) hemolysin is the main pathogenicity element because it has hemolytic, neurotoxic and dermonecrotic action (Berube and Wardenburg, 2013, Olivia and Subtil 2014 and Yadav *et al.*, 2015). There were many reports regarding contribution of α-hemolysin to the pathogenesis of *Staphylococcus aureus* infection, including cell signalling pathways that govern cell proliferation, cytokine secretion, inflammatory responses and cell-cell interactions (Mairpady *et al.*, 2014 and Tkaczyk *et al.*, 2017). Beta (β) hemolysin is an Mg_2^+ dependent sphingomyelinase C, which degrades sphingomyelin in the outer phospholipid layer of the membrane (Linehan *et al.*, 2003). The α and β hemolysin are encoded by *hl*A and *hl*B, gene respectively, and both the genes

are controlled by gene regulatory accessory *agr* (Bownik and Swicki, 2008).

Previous studies report that haemolysin production may be unnecessary to cause mastitis once strains that tested negative for both genes were detected in cattle affected by mastitis (Haveri *et al.*, 2007). However, both haemolysin genes *hl*A and *hl*B were observed in *Staphylococcus aureus* isolates in the present study showing that these genes play an important role in pathogenesis of bovine mastitis.

Coa genotyping

In the present investigation, out of 52 isolates of *Staphylococcus aureus* screened by *coa* gene specific PCR, 43 isolates produced single amplicon of various size. These included 550, 680 and 850 bp size by 19, 11 and 13 isolates, respectively (Fig. 4). The difference of amplification products reflects the variation in *coa* gene length among *Staphylococcus aureus* strains.

The three sizes of amplicons obtained in the present study are in agreement with findings of Sanjiv *et al.* (2008) and Upadhyay *et al.* (2010) who recorded 600, 680 and 850 bp *coa* amplicons using similar primer in *Staphylococcus aureus* isolated from bovine mastitis. However, in contrast to this, four sizes of 600, 680, 710 and 850 bp *coa* amplicons were recorded by Khichar *et al.* (2012), whereas Saei *et al.* (2009) recorded five sizes of 490, 570, 680, 780 and 850 bp amplicons in *Staphylococcus aureus* isolated from bovine mastitis using similar primer. The reason behind this polymorphism is unclear, but it is likely to be caused by the insertion or deletion mutations of some portions in 3' end region of *coa* gene that resulted in a change of the gene size and probably also the antigenic properties of the enzyme. This region of the gene may have an important role in antigenic variation (Saei *et al.*, 2009).

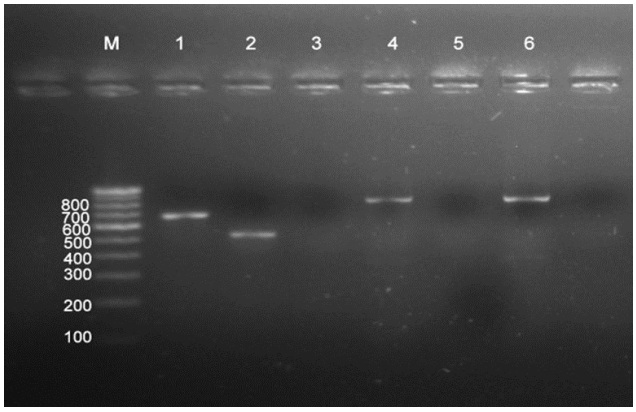

Figure 4: PCR amplification of coa gene of *Staphylococcus aureus* isolates
Lane M : 100 bp DNA marker
Lane 1 : 680 bp PCR product
Lane 2 : 550 bp PCR product
Lane 3 & 5 : Negative sample
Lane 4 & 6 : 850 bp PCR product

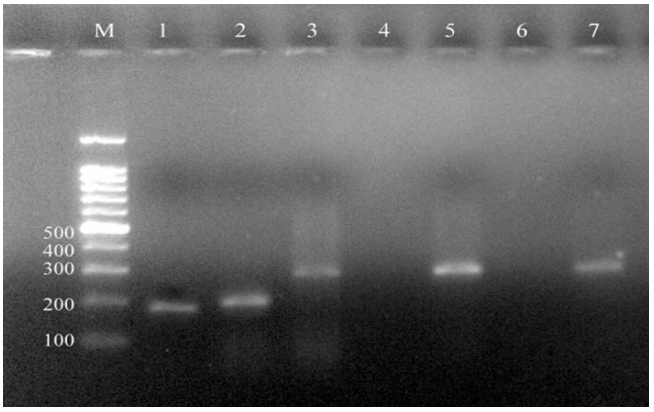

Figure 5: PCR amplification of spa gene of *Staphylococcus aureus* isolates
Lane M : 100 bp DNA marker
Lane 1 : 180 bp PCR product
Lane 2 : 206 bp PCR product
Lane 3, 5 & 7 : 270 bp PCR product
Lane 4 & 6 : Negative sample

The *coa* gene PCR amplification of 43 *Staphylococcus aureus* isolates revealed 3 *coa* PCR types. The most prevalence (44.19%) *coa* gene type was 510 bp length. In line with the present findings, Effendi *et al.* (2019) also reported that most prevalence (50.00 %) *coa* gene type was 510 bp length. In contrast to the present findings, Saei *et al.* (2009) and Katsuda *et al.* (2005) showed that 850 bp and 580 bp *coa* types can be found most prevalent in *Staphylococcus aureus* isolates from milk samples, respectively. The variation in result might be due to predominant types of *Staphylococcus aureus* that vary in different areas and they may be more resistant to neutrophil bactericidal activities than that of the rare types which indicates that they may have different features that help them to survive host immunity mechanism (Su *et al.*, 1999).

From all the isolates, only a single *coa* gene amplicon was obtained in the present study, which is in agreement with Kalorey *et al.* (2007), Upadhyay *et al.* (2010) and Khichar *et al.* (2012) also reported only a single *coa* gene amplicon in *Staphylococcus aureus* isolates obtained from bovine mastitis. However, in contrast to the present findings, da

Silva (2005), Aslantas *et al.* (2007) and Karahan and Cetinkaya (2007) reported the presence of two amplicons of *coa* gene in *Staphylococcus aureus* recovered from bovine mastitis. Goh *et al.* (1992) opined that the variable band size and number observed in *coa* gene-based PCR might have been attributed to the presence of structurally different gene forms of coagulase in *Staphylococcus aureus* allowing one strain to produce one or more of these variants. This might be a plausible explanation for the only one band of different size obtained in the current study.

Moreover, during the study, five isolates which were detected phenotypically by tube coagulase test as coagulase negative were genotypically identified as coagulase positive by *coa* gene-based PCR which corroborated the findings of De Moura *et al*. (2012), Gharib *et al.* (2013), Hamza *et al.* (2015) and Javid *et al.* (2018). These findings emphasize the use of molecular methods in the identification and detection of *Staphylococcus aureus* (Hamza *et al.*, 2015).

The *coa* gene at its 3' end contains a series of 81 bp repeats in tandem which may vary in numbers leading to polymorphism (Phonimdaeng *et al.*, 1990). Scientists have made use of this property of coagulase gene in differentiation of *Staphylococcus aureus* strains for various purposes. According to Aarestrup *et al.* (1995), this technique can be used in epidemiological investigations of *Staphylococcus aureus* mastitis because it is easy to analyse coagulase gene polymorphisms among a large number of strains and the multiple distinct polymorphic patterns generated.

spa genotyping

In detection of X-region of protein A in *spa* gene by PCR, 18 (34.61 %) isolates *Staphylococcus aureus* out of 52 were found positive in which single amplicons of 180, 206 and 270 bp with calculated number of 6, 7 and 10 repeats, were detected indicative of polymorphisms of *spa* gene. (Fig. 5) The amplicon of 270 bp was detected in 8 (15.38 %) isolates followed by 160 bp amplicon in 6 (11.53 %) and 243 bp amplicon in 4 (07.69%) isolates. The amplicon sizes obtained in the present study is almost similar to those reported by Salasia *et al.* (2004) who obtained amplicons of 100, 150, 200, 230, 240, 250, 270, 290 and 340 bp sizes from 35 *Staphylococcus aureus* from milk samples of cattle. Similar to the present findings, Khichar *et al.* (2012) reported 206, 243, 262, 277, 292, 306 and 339 bp with calculated numbers of 7, 8, 9, 10, 10, 11 and 12 repeats, respectively.

Similarly, Annemuller *et al*. (1999) and Karahan *et al*. (2011) also carried out *spa* typing of *Staphylococcus aureus* strains isolated from bovine mastitis and recorded six *spa* types with amplicons varying between 120 to 300 bp and nine *spa* types with amplicons ranging from 100 to 320, respectively. Contrary to the results in the present study, only uniform amplicons of 300 bp size were obtained by Suleiman *et al*. (2012) in 20 isolates of *Staphylococcus aureus* from bovine mastitis. We did not obtain *spa* amplicons in thirty-four (65.38%) isolates. Such *spa* negative *Staphylococcus aureus* isolates have earlier been reported by some workers. In an investigation out of 94 isolates of *Staphylococcus aureus* collected from raw milk from four dairy farms of Brazil, Santos *et al.* (2014) recorded 50 (54.20%) isolates positive for *spa* gene. The absence of *spa-X* region gene has also been reported by Momtaz *et al.* (2010) from bovine clinical and subclinical mastitis; by Salem-Bekhit *et al.* (2010) in bovine mastitis isolates; by Shakeri *et al.* (2010) in healthy carriers and human patients and by Bhati *et al.* (2016) in bovine subclinical mastitis. This result might be attributed to either *spa* mutation occurred or *spa* was absent in these strains. The size polymorphisms in the X-region of *spa* gene that we found in the present study were consistent with the results of Frenay *et al.* (1996); Stephan *et al.* (2001) and Fournier *et al.* (2008) who also reported polymorphisms in the X-region of *spa* gene in *Staphylococcus aureus* obtained from bovine mastitis. Frenay *et al*. (1994) reported an association between the potential distribution of *Staphylococcus aureus* and the number of repeat units. They defined the isolates with more than seven repeat units are considered as an epidemic isolate and with seven or fewer repeat units are considered as non-epidemic isolates. In the present study, out of 18 *spa* positive isolates, 44.44 per cent *Staphylococcus aureus* have more than 7 repeats and therefore our isolates were of an epidemic origin. The further characterisation based on marker or on genetic level might help in development of future vaccines for the control of staphylococcal mastitis in animals.

Among different cell surface proteins produce by *Staphylococcus aureus*, *spa* (*sap*-X) is important and regarded as appropriate target to determine the difference among *Staphylococcus aureus* isolates in a very short period (Reinoso *et al.*, 2008 and Karahan *et al.*, 2011). Protein A produced by *Staphylococcus aureus* is one of the important virulence factors. This protein is used as anchor or tether between organism and the host mucosal cells. Protein A is encoded by *spa* gene which is composed of two functionally distinct regions: N-terminal Fc binding region and

C terminal cell wall binding region designated as X-region. The X-region contains a varying number (between 3 and 15) of small repeat units of 21-27 bp with most repeat units consisting of 24 bp (Frenay *et al.,* 1996) and is highly polymorphic. The amplification of this region thus produces amplicons of variable sizes depending on the number of repeats and this property of polymorphism in X-region of *spa* gene has been utilised for differentiating *Staphylococcus aureus* strains. The variability and stability of this gene indicate that the sequence analysis of *spa* gene can be used as an alternative system to the molecular typing of *Staphylococcus aureus* isolates.

Conclusion

Findings of the present study revealed the presence of virulence genes in S. aureus isolated from bovine mastitis. This is an alarming situation so attention must be paid toward implementation of new ways for effective prophylaxis, control, and treatment of such infections in the dairy farms. Further, it is concluded that PCR assays can be used as rapid and sensitive diagnostic tools to detect virulence factors of *S. aureus* that help in detection of severity of infection, distribution and stating preventive and control strategies.

Acknowledgement

We are thankful to the Dean and the Principal, College of Basic Science and Humanities and Director of Research, S. D. A. U., Sardarkrushinagar for providing necessary facilities to carry out present research works.

Conflict of Interests

There is no conflict of interest.

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