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Polymorphism Analysis of spa Gene of *Staphylococcus aureus* isolated from Bovine Mastitis

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Abstract

Protein A is an important virulence factor of Staphylococcus aureus encoded by the spa gene. The presence of repeatable areas and the highly polymorphic nature of the spa gene, make it a good tool for epidemiologic investigation. The present study was undertaken to determine the spa gene variation in Staphylococcus aureus. A total of 55 S. aureus strains were isolated from cattle and buffaloes with clinical and subclinical mastitis. All the isolates were tested for the presence of spa gene of which 32 (58.18 %) isolates were found positive. Variable amplicon sizes of 160, 243, 270, 296, and 306 bp with calculated numbers of 5, 8, 10, 10 and 11 repeats were detected among 2 (6.25 %), 9 (28.13 %), 8 (25.00 %), 2 (6.25 %) and 11 (34.38 %) isolates, respectively. Furthermore, based on the calculated number, 52.63 percent of cattle Staphylococcus aureus and 58.82 percent of buffalo Staphylococcus aureus have more than 7 repeats. In conclusion, polymorphism of the spa gene was observed in S. aureus isolated from clinical and subclinical mastitis.

Keywords: Bovine Mastitis, PCR, *Staphylococcus aureus*, spa Gene.

Introduction

Milk is a fundamental dietary staple globally, and the dairy industry plays a crucial role in animal husbandry. However, mastitis in dairy cows poses a significant challenge to the advancement of this industry. It stands out as one of the most prevalent diseases, affecting approximately 40 % of the dairy herd. The economic consequences stemming from mastitis infection are substantial, including reduced milk production, compromised milk quality, increased culling rates, and higher treatment expenses. Statistics indicate that mastitis causes economic losses exceeding €185 per cow annually within the European Union alone and up to $35 billion globally (Otero *et al.*, 2024). *Staphylococcus aureus* is the most significant contributor to subclinical and clinical bovine mastitis (Tegegne *et al.,* 2021). It is the second most common source of food poisoning in people because it can produce several enterotoxins in milk and dairy products. Clinical *S. aureus* mastitis can range in severity from mild, which only manifests itself as changes in milk, to per acute gangrenous mastitis, which results in necrosis of the affected mammary quarter, severe systemic symptoms, and sometimes even death of the cow. (Åvall-Jääskeläinen *et al.*, 2021)

The success of *Staphylococcus aureus* as a pathogen is attributable, in part, to the diverse range of virulence factors produced (Gordon and Lowy, 2008). They facilitate the invasion and colonization of host tissue, evasion of the hosts' immune defense mechanisms, aid in the acquisition of nutrients, and dissemination of the bacteria within the host tissue (Haveri *et al.,* 2008). Among the vast array of virulence factors, Protein A is a virulence factor with a molecular weight of 42KD. It is considered one of the important virulence factors in the development and severity of mastitis (Mitra *et al.,* 2013). It is covalently anchored to the peptidoglycan of *Staphylococcus aureus*. This protein is encoded by the *spa* gene which has been shown to have a high degree of variability in size (Brandt *et al.,* 2013). The repeated part in the *spa* gene is located at the 3' end and identified as X region; the repetitive part of region X consists of up to 12 units each with a length of 24 nucleotides. The number of these 24 bp repeats varies among different strains of *Staphylococcusaureus* and hence can be used as a molecular tool in studying the genetic diversity among the strains of *S. aureus* (Strommenger *et al.,* 2008). Given the above facts, the present investigation aimed to study the polymorphism of the *spa* gene (X-region) in the *Staphylococcus aureus* strains of bovine origin.

Materials and Methods

Collection of Milk Samples

A total of 165 milk samples from suspected cases of clinical mastitis in cows (n = 94) and buffaloes (n = 71) from North Gujarat were collected aseptically in sterilized vials. In addition, a total of 256 milk samples from cows (n = 198) and buffaloes (n = 58) from North Gujarat were also collected aseptically and screened for sub-clinical mastitis (SCM) by California Mastitis Test (CMT).

Isolation and Identification of Staphylococcus aureus

Positive milk samples of sub-clinical and clinical mastitis were processed for isolation of *Staphylococcus aureus*. Milk samples were inoculated on Nutrient Agar plates by spreading heavy inoculums of thoroughly mixed milk. The plates were incubated at 37°C for 24 hours. Thereafter, colonies showing golden yellow pigmented or white colony colour indicative of presumptive *Staphylococcus* were transferred to Mannitol Salt Agar which is considered a selective medium for *Staphylococcus*. The colonies of *Staphylococcus*, forming yellow and red colouration indicative of mannitol fermentation and non-fermentation, respectively were transferred to Nutrient Agar slants for further identification. Further identification of these presumptive staphylococcal colonies was first based on conventional methods including Gram stain staining, colony morphology, catalase test, and oxidase test as per the methods described earlier (Cowan and Steel 1974). The isolates were further genotypically confirmed by *Staphylococcus aureus* specific sa442 gene-specific polymerase chain reaction (PCR) using forward primer (5′-AATCTTTGTCGGTACACGATATTCTTCACG-3′) and reverse primer (5′-CGTAATGAGATTTCAGTAGATAATACAACA-3′) as per the method described by Martineau *et al.* (1998).

Amplification of spa Gene

The amplification of *spa* gene encoding protein-A was done as described by Frenay *et al.,* 1996 with slight modifications using 5′-CAAGCACCAAAAGAGGAA-3′ (F) and 5′-CACCAGGTTTAACGACAT-3′ (R) primers.

The genomic DNA of *Staphylococcus aureus* isolates was extracted by QIAamp DNA Mini kit as per the standard protocol outlined in the manufacturer's manual. Template DNA (5.0 µl) was added to the PCR mixture (total volume of 25 µl) containing 12.5 µl of 2X PCR Master Mix, 1.0 µl (10 pmol) of each forward and reverse primers, 5.5 µl nuclease-free water. The PCR was performed in Nexus Mastercycler (Eppendorf) using following cycling parameters: Initial denaturation at 94°C for 5 min; 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min; with a final extension at 72°C for 5 min. The amplicons so generated by PCR amplification were resolved on agarose gel electrophoresis (Bangalore GeNei). Five microliter of PCR product was mixed with 2 µl of 6X gel loading dye. The mixture was loaded on the well and electrophoresed on 1.5 per cent agarose gel along with 100 bp DNA Ladder (GeneRuler-Fermentas) at constant 80 V for 45 minutes in 1X TAE buffer. The gel was then visualized under Gel Documentation System (DNR Bio-imaging System). Calculation of a number of tandem repeats (N) in PCR amplified *spa* gene product was done using the formula given by Frenay *et al.*, 1996. Mathematically, formula is given as:

$$N = \frac{\text{Size of amplified } spa \text{ gene product} - \text{Size of Primers (Forward + Reverse)}}{24}$$

Results and Discussion

Bovine mastitis is the most common and quite damaging disease throughout the world. It is a versatile disease in milch animals and is caused by pathological, genetical, physiological, or environmental factors. Despite the concerted efforts to control or reduce the incidence of mastitis for decades, it remains a major threat to the dairy industry causing huge economic loss. In the present study, out of 256 milk samples screened, 34 samples were detected positive for SCM, which included 25 samples from cows and 9 samples from buffaloes indicating an incidence of sub-clinical mastitis of 12.63 percent in cows and 37.50 percent in buffaloes. Thirty-four samples were found positive for sub-clinical mastitis by the California mastitis test and 165 samples of clinical mastitis were inoculated on Nutrient Agar for primary isolation. A total of 55 *Staphylococcus aureus* isolates which included 38 from Cows and 17 from buffaloes were isolated and identified based on cultural and biochemical properties.

Although *Staphylococcus aureus* can be identified by its phenotypic characteristics, often cultural and biochemical tests are cumbersome and time-consuming. Furthermore, the biochemical techniques showed vagueness in results due to flexibility in bacterial nature and growth depending on the laboratory environment (Stepan *et al.*, 2004). In addition, *Staphylococcus aureus* has many colony variants and shows variation in phenotypic expressions (Qureshi and Kataria, 2012). Hence, to overcome the limitations of cultural and biochemical methods, molecular techniques have been described to obtain an accurate and rapid confirmation (Morandi *et al.*, 2009).

For the identification of *Staphylococcus aureus,* various PCR-based detection systems were developed targeting specific genes coding for different traits *viz*., thermostable nuclease, enterotoxins, TSS-toxins or penicillin-binding proteins, but these were not sufficiently reliable to detect all strains as these genes are not present in all strains of *Staphylococcus aureus*. To improve this situation, Martineau *et al*. (1998) identified a 442 bp chromosomal DNA fragment which is specific for *Staphylococcus aureus*. This DNA-based test provides a novel diagnostic tool for the identification of *Staphylococcus aureus* infections. Therefore, sa442-based PCR was used to confirm *Staphylococcus aureus* in the present study. All 55 isolates produced 108 bp amplicon of *Staphylococcus aureus* specific sa442 gene confirming them as a *Staphylococcus aureus.* Protein A produced by *Staphylococcus aureus* is one of the important virulence factors used as an anchor or tether between the organism and the host mucosal cells. Protein A is encoded by the *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 of 3 to 15 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 utilized for differentiating *Staphylococcus aureus* strains.

In the present study, 32 (58.18 %) isolates out of 55 isolates of *Staphylococcus aureus* were found positive for X-region of Protein A in *spa* gene showing single amplicons of variable sizes *viz.*,160, 243, 270, 296 and 306 bp with calculated number of 5, 8, 10, 10 and 11 repeats, respectively (Figure 1). Further, out of 38 isolates from cattle, 21 strains produced *spa* amplicons, whereas 17 isolate did not produce any amplified product. Eight isolates produced amplicon of 243 bp, seven produced amplicon of 306 bp, four produced 270 bp amplicon, and one each isolate produced amplicon of 296 bp and 150 bp with calculated number of 8, 11, 10, 10 and 5 repeats, respectively. The

spa gene amplicons produced by 11 isolates from buffalo were obtained of size of 160 bp, 243 bp and 296 bp produced by each one isolate, 270 bp and 306 bp produced by each of four isolates with calculated number of 5, 8, 10, 10 and 11 repeats, respectively. Species-wise details are given in Table 1.

Table 1: Detection of polymorphism in X-region of protein A (*spa*) gene of *Staphylococcus aureus* isolates

Sr. No	Size of amplicon (bp)	Total isolates (Cow)	Total isolates (Buffalo)	No. of tandem repeats
1	160	1 (4.76 %)	1(09.09%)	5
2	243	8 (38.09 %)	1(09.09%)	8
3	270	4 (19.05 %)	4 (36.36%)	10
4	296	1 (4.76 %)	1(09.09%)	10
5	306	7(33.33 %)	4(36.36%)	11
Total		**21**	**11**	

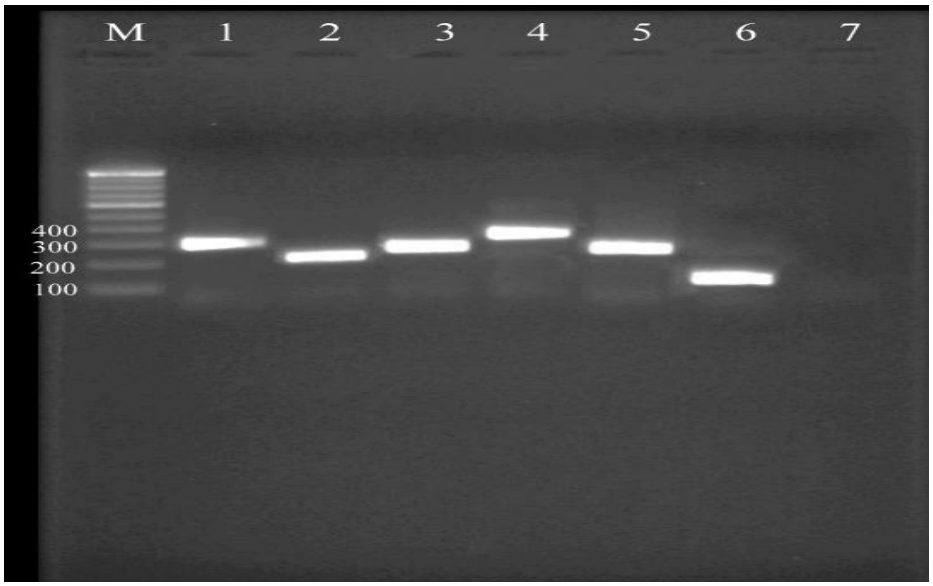

Figure 1 : PCR amplification of *spa* gene of *Staphylococcusaureus* isolates

Lane M : 100 bp DNA Marker Lane 1 : 296 bp PCR product
Lane 2 : 243 bp PCR product Lane 3& 5 : 270 bp PCR product
Lane 4 : 306 bp PCR product Lane 6 160 bp PCR product
Lane 7 : Negative Control

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 varied 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 *S. aureus* from bovine mastitis.

In the present study, most of the isolates possessed 5, 8, 10and 11 repeats of 24 bp in the variable X-region of the *spa* gene which corroborate the findings of Walker *et al.* (1998) and Salasia *et al.* (2004) who detected 11 and 10 to 12 *spa* repeats in most of the *S. aureus* isolates from bovine mastitis, respectively. Contrary to the present findings, Stephan *et al.* (2001) and Annemuller *et al.* (1999) recorded 2 to 4 *spa* repeats in most of the *S. aureus* isolates from bovine mastitis.

The size polymorphisms in the X-region of the *spa* gene 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 the *spa* gene in *Staphylococcus aureus* obtained from bovine mastitis.

In the present study, 23 *Staphylococcus aureus* isolates did not contain the *spa* gene. In line with the present study, the absence of *spa* X-region gene has also been reported by Bekhit *et al.* (2010) and Momtaz *et al*. (2010) in *Staphylococcus aureus* isolates obtained from bovine mastitis. These results might be attributed to either *spa* mutation occurred or *spa* was absent in these strains.

Frenay *et al*. (1994) reported an association between the potential distribution of *S. aureus* and the number of repeat units. They reported that most epidemic MRSA strains harbored more than seven repeats while non-epidemic MRSA strains contained seven or fewer repeats. Considering the above fact, 52.63 percent of cattle *Staphylococcus aureus* and 58.82 percent of buffalo *Staphylococcus aureus* have more than 7 repeats indicating overall 54.55 percent of *Staphylococcus aureus* in the present study was of an epidemic origin. Further characterization based on markers or the genetic level might help in the development of future vaccines for the control of staphylococcal mastitis in animals.

Conclusion

The present study revealed polymorphism in *spa* X-region gene amplicons of *S. aureus* obtained from clinical and subclinical mastitis cases from cattle and buffaloes. A wide degree of polymorphism was observed in the isolates. In the present study, out of 55 *Staphylococcus aureus*isolates 30 were considered to be pathogenic since they possessed seven or more repeats. Based on the variability and stability of the *spa* gene indicate that the sequence analysis of the *spa* gene can be used as an alternative system to the molecular typing of *S. aureus* isolates.

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Contribution by Authors

All co-authors contributed equally.

Conflict of Interests

There is no conflict of interest.

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