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Diwakar
M.V.Sc. Scholar, Apex Centre for Animal Disease Investigation, Monitoring and Surveillance Department of Veterinary Microbiology and Biotechnology College of Veterinary and Animal Science Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

S Choudhary
M.V.Sc. Scholar, Apex Centre for Animal Disease Investigation, Monitoring and Surveillance Department of Veterinary Microbiology and Biotechnology College of Veterinary and Animal Science Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Akriti
Project Associate, Centre for excellence for use of space based technology in animal science, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences Bikaner, Rajasthan, India

T Bhati
Assistant Professor, Department of Veterinary Microbiology and Biotechnology College of Veterinary and Animal Science Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

AK Kataria
Professor & Head, Apex Centre for Animal Disease Investigation, Monitoring and Surveillance Department of Veterinary Microbiology and Biotechnology College of Veterinary and Animal Science Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Correspondence
Diwakar
M.V.Sc. Scholar, Apex Centre for Animal Disease Investigation, Monitoring and Surveillance Department of Veterinary Microbiology and Biotechnology College of Veterinary and Animal Science Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Polymorphism in x-region of *spa* gene in *S. aureus* isolates from goats with clinical mastitis

Diwakar, S Choudhary, Akriti, T Bhati and AK Kataria

Abstract
Staphylococcus aureus is the most frequently mastitis causing bacterial pathogen in dairy milch animals including goats. In the present investigation 34 *S. aureus* from goat mastitis were characterize for x-region of *spa* gene where in amplification of x-region of *spa* gene produced amplicons in 32 isolates of with specific primers whereas two did not produce any amplicon which were considered *spa* negative isolates. Of the 32 isolates 23 produced single amplicon, 8 produced double amplicons and one isolate produced three amplicons. All the 32 isolates were divisible into 19 *spa* types and the size of amplicons obtained were 80, 200, 220, 240, 250,260, 280, 300, 320 and 340bp with a calculated number of repeats of 3, 3, 3, 5, 1, 3, 2, 4, 2 and 2, respectively.

Keywords: *Staphylococcus aureus*, goat, *spa* gene mastitis, protein-A

Introduction
Mastitis is multi-etiological complex but *S. aureus* has been found to be the most important pathogen worldwide in dairy small ruminants [1, 2, 3]. In severe cases staphylococcal infection may progress to gangrene which is characterized by discoloration of udder, abscess development, draining pus and systemic signs of toxemia [4].
Staphylococcal protein A (*spa*) is a membrane-bound exoprotein of bacterial cell wall and is considered an important virulence factor that impairs opsonization by serum complement and phagocytosis of polymorphonuclear leukocytes through binding to Fc region of immunoglobulins [5]. This protein A is encoded by *spa* gene with a polymorphic x-region consisting of a variable number of repeated 24 pairs of nucleotides [6]. This variable number of repeats of gene is being used as an important marker in identification and epidemiological studies. Frenay *et al*. (1996) [7] has reported that the isolates possessing seven or more tandem repeats are pathogenic isolates.
The present investigation was designed to study the polymorphism of *spa* gene (X-region) typing in *S. aureus* isolates from goats with clinical mastitis.

Materials and Methods
Isolation of *S. aureus*
Sampling
A total of 57 milk samples in 5-10 ml amount each, were collected from untreated mastitic goats of non-descript breed belonging to different famers in and around Bikaner city (Rajasthan India). The goats with visible abnormalities in milk (watery milk, clots, pus, blood in milk etc.) and physical changes in udders were considered to have clinical mastitis. The sample collected in sterilized test tubes were immediately transferred to the laboratory on ice for further processing.

Isolation and identification: The organisms were isolated and identified as described [8, 9]. Of the 57 samples, 34 isolates of *S. aureus* were obtained which were further confirmed by 23S rRNA ribotyping [10] using the following species-specific primers, Primer-F: 5'-ACGGAGTTACAAAGGACGAC-3' and Primer R: 5'AGCTCAGCCTTAACGAGTAC-3'.

Amplification of *spa* gene
The amplification of *spa* gene x-region was done as described [7] with some modifications using 5′-CAAGCACCAAAAGAGGAA-3′ (F) and 5′-CACCAGGTTTAACGACAT-3′ (R) primers. The reaction was performed in 0.2 ml thin-walled PCR tubes.

The reaction mixture (total volume 25 µl.) was prepared by mixing: 11.9 µl deionised water, 5.0 µl 10x Buffer, 2.5 µl MgCl2, 0.8 µl dNTP-mix (10mM), 0.8 µl of each primer pM/µl), 0.2 µl TaqDNA polymerase (5U/µl) and 3 µl template DNA (25ng/ µl). Amplification was carried out in Veriti thermal cycler (Applied biosystem) as follows: initial 34 cycle of amplification (denaturation at 94 °C for 60s, primer annealing at 55 °C for 60s and primer extension at 70 °C for 60 s), and final extension at 72 °C for 5 min. The PCR products were resolved in 1.2% agarose gels prepared in 1x TBE buffer containing 0.5 µg/ml of ethidium bromide. 100bp ladder as molecular marker. The amplification products were electrophoresed for 60 min at 100 V. The gel was then visualized under gel documentation system (ENDURO GDS).

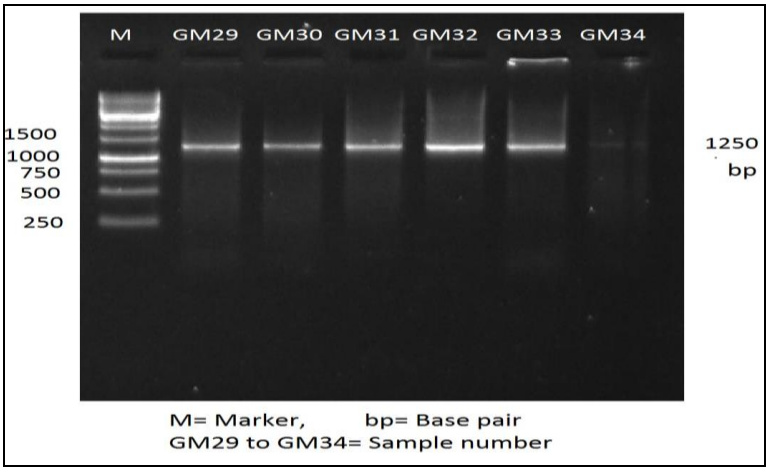

Fig 1: Agarose gel electrophoresis of amplicons of 23S rRNA ribotyping of *S. aureus* isolates obtained from goats with clinical mastitis

Results

The ribotyping produced an amplicon of 1250 bp in all isolates confirming them to be *S. aureus* (Fig. 1). Protein A is encoded by *spa* gene and is one of the important virulence factors involved in the staphylococcal pathogenesis. The amplification of *spa* gene (X- region) produces amplicons of variable sizes depending on the number of 24 bp tandem repeats correlates with the virulence level of the strains. The amplification of this region thus produces amplicons of variable sizes depending on the number of repeats. In the present investigation amplification of X-region of *spa* gene produced amplicons in 32 isolates of *S. aureus* with specific primers whereas two did not produce any amplicon which were considered *spa* negative isolates. Of the 32 isolates 23 produced single amplicon, 8 produced double amplicons and one isolate produced three amplicons (Fig. 2)

In the present study a wide range of *spa* gene amplicons were obtained with goat mastitis isolates. The size of amplicons varied from 80 to 340 bp. On the basis of *spa* (x-region) gene typing all the isolates were divisible into 19 spa types (Table 1).

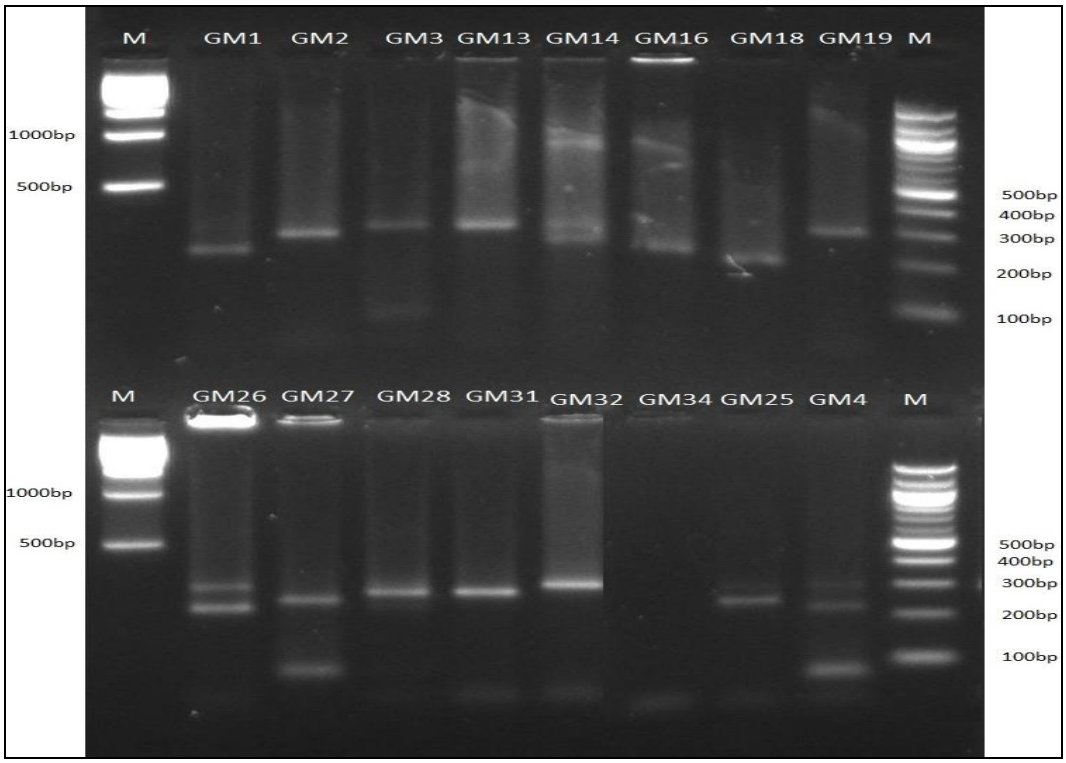

Fig.2: Agarose gel electrophoresis of amplicons of *Spa* gene (X-region) of *S. aureus* isolates obtained from goats with clinical mastitis.

Discussion

The ribotyping produced an amplicon of 1250 bp in all isolates confirming them to be *S. aureus*. This method of ribotyping for identification of *S. aureus* has been used by various workers in different localities and laboratories Salasia *et al.* (2004) [11], Bhati *et al.* (2016) [12] and Choudhary *et al*. (2018) [13]. In our study, 32 of the isolates produced *spa* amplicons which were divisible into 19 spa types. Salasia *et al.* (2004) [11], Bystron *et al*. (2009) [14], Yadav *et al*. (2015) [15], Bhati *et al.* (2016) [12] and Choudhary *et al*. (2018) [13] reported a wide range of amplicon sizes obtained with amplification of *spa* (x-region) gene in *S. aureus* of different origins. This typing has been utilized by many of the workers to study the epidemiological spread of the isolates Lange *et al*. (1999) [16], Stephan *et al*. (2001) [17], Singh *et al*. (2011) [18] and Khichar *et al*. (2014) [19]. On the contrary there are few reports where only single amplicon size was detected in all the isolates in their study *viz*. Abdeen *et al.* (2015) [20] detected single *spa* gene amplicon of 226bp size in 35 strain of *S. aureus* from clinical mastitis in cattle. Similarly, Momtaz *et al.* (2010) [21] reported all the *S. aureus* isolates of bovine mastitic milk origin with *spa* gene to produce amplicon of 320 base pairs only. Uniform amplicons of 300 bp size were obtained by Suleiman *et al*. (2012) [22] in 20 isolates of *S. aureus* from subclinical bovine mastitis.

Table 1: *spa* gene (X- region) polymorphism in *S. aureus* isolates from goats with clinical mastitis.

S. No	Isolates numbers	Total isolates	*spa* (X- region) gene amplicon size (bp)	No. of tandem repeats
	GM1, GM16	2	250	8
	GM2, GM22, GM29, GM32, M33	5	300	11
	GM3, GM13, GM19	3	340	12
	GM4	1	300, 220, 80	11,7,1
	GM5, GM11,GM12, GM21, GM23	5	240	8
	GM6	1	280, 240	10, 8
	GM7, GM28, GM31, GM 24	4	260	9
	GM8	1	320, 240	11, 8
	GM9	1	320	11
	GM10	1	80	1
	GM14	1	340, 280	12, 10
	GM15, GM34	2	Absent	-
	GM17	1	260, 200	9, 6
	GM18	1	220	7
	GM20	1	200	6
	GM25	1	300, 240	11, 8
	GM26	1	300, 220	11, 7
	GM27	1	240, 80	8, 1
	GM30	1	260, 200	9, 6

In our study we observed eight isolates to produce double and one to produce three amplicons in each. Many of the workers have reported this kind of results. Choudhary *et al*. (2018) [13] observed nine isolates which produced two amplicons. Bhati *et al.* (2016) [12] reported one of the 38 isolates to produce double amplicons.

Of the 34 isolates 23 produced single amplicon, 8 produced double amplicons and one isolate produced three amplicons whereas two (5.88%) did not produce any amplicon which were considered *spa* negative isolates. Momtaz *et al.* (2010) [21] also reported 19.76% (17 of 86) *S. aureus* isolates of bovine mastitic milk origin without *spa* gene. Similarly, Santos *et al.* (2014) [23] observed that 53.19% of 94 *S. aureus* isolates obtained from raw milk in dairy farms of Brazil did not carry *spa* gene.

The most common amplicon size obtained in our study was of 240 bp which was detected in nine isolates (five single and four in double) followed by 300 bp amplicon which was detected in eight isolates.

In the present study of the 34 isolates 30 were considered to be pathogenic since they possessed seven or more repeats. Khichar *et al.* (2014) [19] carried out characterization of 28 *S. aureus* of cattle mastitis origin all of which were also reported to be highly pathogenic on the basis of number of repeats. This is based on reports by Frenay *et al.* (1996) [7] who suggested that the isolates possessing seven or more tandem repeats were pathogenic isolates. On the other hand, no correlation was reported between tandem repeats and pathogenicity of the isolates by Nashev *et al*. (2004) [24] from humans; Kuzma *et al*. (2005) [25] and Jakubczak *et al*. (2007) [26] in isolates from mastitc cows and Kurlenda *et al*. (2010) [27] in human isolates.

Conclusion

The present study revealed a wide range of polymorphism in *spa* gene amplicons of *S. aureus* obtained with goat mastitis. Based on the number of repeats, 30 isolates were considered pathogenic since they possessed more than seven repeats. The number of repeats along the X region of the *spa* gene correlates with the virulence level of the strains.

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