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Research Article

MOLECULAR CHARACTERIZATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) ISOLATED FROM BOVINE MASTITIS IN AND AROUND KOLKATA, INDIA

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ABSTRACT: The present investigation was carried out on 118 milk samples collected from suspected clinical and subclinical mastitis affected indigenous and cross-bred cattle in and around Kolkata and North 24 Parganas districts. A total of 38 samples were positive for *Staphylococcus aureus*. This indicates that considerable proportion of cattle with intramammary infection was colonized with *S. aureus.* However, sizeable populations of those pathogens (15 of 38) were methicillin resistant (MRSA) by phenotypic detection method. Methicillin resistance gene (*mecA*) was detected from 6 samples out of those 15 samples. Overuse or inadvertent use of antibiotics may be the cause of development of such resistant strains in livestock. This may have huge public health significance as these resistant strains may enter the food chain and infect the consumer with consequences of life-threatening infections.

Key words: Bovine mastitis, *Staphylococcus aureus*, Methicillin resistance.

INTRODUCTION

The attachment of human with animals and birds has given a pace in emerging and increasing infectious diseases. It is estimated that over 60% of the emerging human pathogens have come from the animals (Cutler *et al*. 2010).

Intensive animal farming practices facilitated the emergence of new pathogens and their transmission to humans. Stable settlements and proximity to the animals caused the pathogens to thrive and spread between the animals and humans and the infectious diseases become prominent in influencing the life and death (Porter 1997).

In India, the economic losses due to mastitis have increased about 115 folds in the last five decades and presently the loss due to mastitis is to the tune of Rs.71.65 billion per annum. Since mastitis also affects the milk quality, its consequences are not restricted only to the farm but it also expands beyond the dairy farm. The increasing concerns of the consumers about the antimicrobial residues are the antimicrobial resistance, milk quality and animal welfare which further demand for proper policies for effective prevention and control of the mastitis as reported in NDRI news (Srivastava 2012).

Major mastitis pathogens such as *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae* and coliforms are usually considered virulent and damaging to the udder (Reyher *et al.* 2012).

Methicillin is a narrow spectrum beta-lactam antibiotic of the penicillin class developed in 1959 was previously used to treat the infections caused by *S.aureus*. The presence of the ortho-dimethoxyphenyl group, directly attached to the side chain of the carbonyl group of the penicillin nucleus facilitates the β-lactamase resistance.

S. aureus has the characteristic ability to develop rapid resistance virtually to any antibiotic drug used for treatment. Penicillin-resistant strains of *S. aureus* appeared as early as in 1940, but for many years these remained susceptible to the β-lactamase-stable Penicillins. Resistance to methicillin – that indicates the resistance to all beta-lactam agents – was first reported in 1961, also

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marked the appearance of Methicillin Resistant *S. aureus* (Pantosti 2012).

Methicillin resistant *Staphylococcus aureus* (MRSA) was also identified in mastitis milk samples of buffalo reared by marginal farmars of northern India by Biswas *et al.* (2018).

Methicillin resistance is due to the acquisition of the *mecA* gene, that encodes a new protein designated as PBP2a, belonging to a family of enzymes necessary in building the bacterial cell wall. PBP2a has a very low affinity for β-lactam antibiotics and confers the resistance to methicillin and other beta-lactams (Pantosti *et al*. 2007). The *mecA* gene is located on a mobile genetic element, named as staphylococcal cassette chromosome *mec* (SCC*mec*) (Katayama *et al.* 2000). Different types of SCC*mec* can be distinguished on the basis of different key elements present, which are called the *mec* gene complex, comprising *mecA* and its regulatory genes *mecI* and $mecR_1$ (Ito *et al*. 2001).

Therefore, it is very necessary to study and characterize MRSA in every part of the country, especially in and around Kolkata where incidence of mastitis is quite common.The present study was undertaken to characterize methicillin-resistant *S. aureus* isolated from clinical cases of bovine mastitis and to determine the genetic mechanism of beta-lactam resistance of the *S. aureus* isolates.

MATERIALS AND METHODS

Collection of samples

For this study the milk samples of suspected clinical and subclinical mastitis affected indigenous and crossbred cows were collected from Ganganagar, Duttapukur, Gobardanga, Guma of North 24 Pargana district and Cattle ward, WBUAFS, Kolkata. A total no of 118 cases were screened for mastitis from the different places mentioned above. After the collection, the samples were brought to IVRI, Eastern Regional Station, Kolkata for thorough study.

Diagnosis of clinical cases of mastitis were done by visualization and palpation of the udder for presence of symptoms and screening of the milk samples by observation, pH detection (with pH meter, Hanna Instruments, ISO-9001) as well as California Mastitis Test (using 'Masti Check'® California Mastitis Reagent, manufactured by RFCL Limited, Okhla Industrial Area Phase-I, New Delhi- 110020, India).

A sample of milk from each lactating quarter of each affected animals of the milking herd was collected aseptically and processed separately in the laboratory. The milk samples were transported to the laboratory on the same day in an ice box collected in peptone water having 7% salt concentration as a carrying media.

Bacteriological culture and identification

The milk collected in peptone water in the sterile vial was shaken well for proper mixing and then centrifuged at 4000 rpm for 10 minutes. After that the supernatant fat layer was discarded and the sediment was subjected to test. Two to three loopful of the sediment was inoculated into the sterile test tube containing sterile nutrient broth and incubated at 37^0C for overnight.

A loopful was taken from the incubated broth and was streaked on Mannitol-salt agar (MSA) plates and further being incubated at 37^0C for 24 hours to detect the presence of *Staphylococcus* sp. A yellow and a pink colony on (MSA) plates probably indicate *Staphylococcus aureus* and *Staphylococcus epidermidis* respectively. One golden yellow colony of each Mannitol-salt plate was characterized through bacteriological standard techniques as per the methods of Schalm *et al.* (1971), Wilson and Miles (1974) and Cruickshank *et al*. (1980) for isolation and identification of *S. aureus*. These tests are- Catalase test, Oxidase test, Glucose & Mannitol fermentation and Lecithinase reaction.

Confirmation of *Staphylococcus aureus* by PCR

In this step, *nuc* gene was checked by PCR method as *nuc* gene bearing *Staphylococcus* are virtually *Staphylococcus aureus* (Fang *et al*. 2003). To perform PCR, DNAs from the positive samples were extracted.

DNA extraction

Before DNA extraction, a golden yellow single colony taken from each Mannitol salt agar plates was streaked on Mueller-Hinton agar plates with a sterile loop for growth of the bacteria and incubated for 24 hours at 37^0C. On the next day with a sterile loop, a loopful culture was transferred to 1.5 ml microfuge tubes aseptically. Each tube contained 200 µl of nuclease free water and it was mixed well by vortexing @ 4000 rpm for 1 minute. The suspension was boiled at 100^0C in dry bath for 10 minutes and then chilled immediately by keeping at ice bath for 5 minutes and then centrifuged @4000 rpm for 10 minutes. The supernatant portion was subjected to PCR for gene screening (Stegger *et al.* 2012).

Amplification of *nuc* gene by PCR

The *nuc* gene was amplified using specific primers as mentioned in Table 1 (Fang *et al*. 2003).

Antibiotic sensitivity test (AST) by single disc diffusion method for detection of MRSA

Antibiotic susceptibility tests were performed on all

Table 1. Primer sequences for amplification of the *nuc* gene in *S. aureus* isolates.

Gene	Primer Sequence (5' - 3')	Size of amplicon (bp)
nuc	F- GCG ATT GAT GGT GAT ACG GTT R- CAA GCC TTG ACG AAC TAA AGC	272

Amplification was evaluated in 1.5% gel electrophoresis using UV trans- illuminator.

S.aureus isolates to determine their antibiotic-resistance profiles in the Mueller Hinton agar. Susceptibilities of the isolates to Methicillin and Cefoxitin (6 µm in diameter) were determined. Diameters of zone of inhibition were measured and the value obtained from the National Committee on Clinical Laboratory Standards (NCCLS) was used to interpret the results obtained. *S. aureus* isolates were then classified as resistant, intermediate resistant and susceptible to a particular antibiotic (Table 2).

Detection of drug resistant genes of *S. aureus*

Detection of *mec* A gene by PCR is considered as the best standard procedure. DNA extractions from positive isolates were performed and *mec*A gene was amplified using specific primers (Table 3). Amplification was evaluated in 1.5% gel electrophoresis and gel revealed that *mec* A primer was yielding at 162 base pair (Stegger *et al.* 2012).

Molecular Characterization of MRSA isolates

***spa* Typing**

The *spa* gene was amplified using specific primers (Table 4) (Harmsen *et al*. 2003).

Gel purification of Genes

An isolate showing distinct band during preliminary screening of a gene in specific PCR was selected. Gene to be purified was first amplified in 6 separate PCR tube containing 25 µl of reaction mixture using DNA of the selected isolate. After PCR amplification, total 150 µl of PCR amplified product was run in 0.9% agarose gel prepared from TAE buffer. Then the band of required amplicon was cut using clean, sharp scalpel with the help of a UV illuminator, and collected in a 2 ml microfuge tube for purification. If the weight of the gel piece is more than 600 mg, gel was cut into small pieces accordingly and kept at - 20 °C for temporary storage. The purification was done using QIAquick® column as per protocol.

Sequencing of the gene

The gel purified products were sent along with the primers for sequencing to Scigenome (India) to get the nucleotide sequencing report. After analysis of sequencing reports the type of gene was assessed.

RESULTS AND DISCUSSION

Prevalence of mastitis

From the 118 suspected cases of mastitis, 91 (77.11%) gave the positive results in pH detection and 78 (66.10%) were found positive in California Mastitis Test (CMT) (Table 5 and Table 6). The pH of milk, normally around 6.6, can increase to 6.8 or 6.9 in mastitic cows. The utility of CMT in detection of mastitis corroborated positively with the observations of Hueston *et al.* (1986) who stated that the overall sensitivity of the CMT for detecting intra-mammary infection is 69.3%.

CMT helps in mastitis therapy program, reduces the risk of antibiotic residues in milk and increases both the quality and quantity of milk produced. It is based on the principle that the addition of a detergent to a milk sample with a high cell count will lyse the cells, release nucleic acids and other constituents and lead to the formation of a 'gel-like' matrix consistency. However, the interpretation can be subjective, and this might result in false positives and negatives (Viguier *et al*. 2009).

Prevalence of mastitis in different breeds

From the 78 positive samples, 55 (70.51%) were from the cross bred cattle and 23 (29.49%) were from the indigenous cows (Table 7).

The present findings showed that the prevalence of both clinical and subclinical mastitis was highest in the cross bred cattle (70.51%) and lowest in the indigenous cattle (29.49%). Similar trend was also observed in an earlier study from West Bengal where subclinical mastitis

Table 2. Status of antibiotic susceptibility of *S. aureus* as per NCCLS.

Antimicrobial agents	Symbols	Conc. per disc (mcg)	Zone of inhibition size (mm)		
			Resistant	Intermediate	Sensitive
Methicillin	MET	5	9	10-13	14
Cefoxitin	CX	30	14	15-17	18

Table 3. Primer sequences for amplification of the respective genes in *S. aureus* isolates.

Gene	Primer Sequence (5' - 3')	Size of amplicon (bp)
*mec*A	F- TCC AGA TTA CAA CTT CAC CAG G R- CCA CTT CAT ATC TTG TAA CG	162

cases were recorded as 62.8%,44.7% and 1.9% in Holstein Friesian, Brown Swiss and Hariana cows respectively (Roy *et al.*1989).

This might be due to high yielding crossbred cows are more prone to the udder infections than the low producing ones as stated by Slettbakk *et al.* (1995) and Radostits *et al.* (2000). The production of a large quantity of milk keeps the glandular tissues more generative and thus become more susceptible to the infections.

Inoculation in solid media

Fifty six positive samples were detected in MSA media after culturing for 24 hrs at 37^0C. One colony of each mannitol-salt plate was characterized through standard bacteriological techniques (Catalase test, Oxidase test, Glucose and Mannitol fermentation and Lecithinase reaction) as per the methods of Schalm *et al.* (1971), Wilson and Miles (1974) and Cruickshank *et al*. (1980). After biochemical tests, only 38 samples were positive in all biochemical tests mentioned before and were taken as *S. aureus* phenotypically (Table 8).

Prevalence of methicillin-resistant *S. aureus*

Out of 38 *S. aureus* isolates,15 (39.47%) were found resistant to methicillin and Cefoxitin. Therefore it was recorded in this study that out of 38 *S. aureus* isolates, 15 were positive for MRSA (Table 9). Similar results were also observed in a study conducted by Rubeena *et al.* (2004) who noted 448 isolates (35.67%) out of 1322 isolates were positive for MRSA.

In *S. aureus*, resistance to penicillin occurs through two ways, by the production of the β-lactamase enzyme and by the development of the *mecA* gene. Majority of *S. aureus* strains now a day produces β-lactamase and thus become resistant to penicillin (Cosimo *et al.* 2004).

Although all the cells in a population of *S. aureus* may carry the *mecA* gene, only a few of the cells will express the gene. Thus, both resistant and non-resistant bacteria can exist in the same culture. This *mecA* expression can be constitutive or inducible and is carried on a mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*) (Enright *et al.* 2002).

The 15 phenotypically MRSA positive samples were also subjected to *nuc* PCR to confirm that they were *S.*

Table 4. Primer sequences for amplification of the *spa* gene in *S. aureus* isolates.

Gene	Primer Sequence (5' - 3')
Spa	F-TAA AGA CGA TCC TTC GGT GA R- CAG CAG TAG TGC CGT TTG CTT

Table 5. Positive results for mastitis in different tests.

Total no. of cases screened	Positive in CMT	Positive in pH detector
118	78	91
Percentage (%)	66.10	77.11

Table 6. List of cases of mastitis in cows from different places in and around Kolkata.

Place	Total no of mastitis cases in cows screened	No. of samples positive in pH detection	No. of samples positive in CMT
Ganganagar (GM)	56	49	41
Duttapukur (DM)	24	22	21
Gobardanga (GOM)	12	7	7
Guma (GUM)	16	9	6
CattleWard, WBUAFS (CM)	10	4	3
Total	118	91	78

Table 7. List of cases of mastitis in cows of different breeds from different places in and around Kolkata.

Places	Total no of positive mastitis cases	No. of crossbred cows with positive results	No. of indigenous cows with positive results
Ganganagar (GM)	41	35	6
Duttapukur (DM)	21	12	9
Gobardanga (GOM)	7	2	5
Guma (GUM)	6	4	2
Cattle Ward, WBUAFS (CM)	3	2	1
Total	78	55	23

Table 8. List of samples positive in all biochemical tests done.

Biochemical tests	Sample no. (positive in all biochemical tests)
Catalase test,	GM-1,2,3,4,5,6,7,8,9,10,13,16,19,21,22,24,26,28,29,32,33,34,37,39
Oxidase test,	DM-1,2,3,5,7,11,17
Glucose and Mannitol	GOM-1,3,5
fermentation and	GUM-4,5
Lecithinase reaction	CM-2,3

Table 9. List of samples found positive as MRSA.

MRSA status	Sample no.
Positive	GM-1,2,3,4,6,7,8,10
	DM-2,5,7
	GOM-5
	GUM-4,5
	CM-2

Table 10. List of sample no. observed as positive for *nuc* gene.

nuc gene	Sample no.
positive	GM- 2, 3, 4, 6, 7, 8, 10
	DM- 2, 5
	GOM-5

Table 11. List of samples observed as positive for *mecA* gene.

mecA gene	Sample no.
Positive	GM- 2, 3, 4, 6, 7, 8

aureus genotypically and 10 samples had given positive results to *nuc* PCR (Table 10, Fig. 1). This can be due to the fact that other *Staphylococcus* sp. which are also methicillin-resistant or mixed infection by different *Staphylococcus* sp.

Detection of resistant gene (*mecA*) by molecular methods

Detection of resistant gene, *mecA* by PCR was considered as the best standard procedure (Oliveira and Lencastre 2002).The presence or absence of *mecA* gene was determined for all confirmed *S. aureus* isolates by simplex PCR as previously described by Jayaratne and Rutherford (1999). Pure yellow colonies obtained from a fresh subculture on Mannitol Salt agar with 6 mg/L oxacillin used as the DNA template for PCR amplification. *mecA* primer was yielding at 162 base pair (bp).Total 6 *mecA* genes were isolated out of 15 MRSA (Table 11, Fig. 2).

The turnaround time (TAT) was taken as the time to detect the methicillin resistance in the *S. aureus* isolates. The TAT excluded the time for the primary growth and identification of the *S. aureus* isolate, which was approximately the same time (18.5 hours) for all methods. The TAT for detection of methicillin resistance in *S.*

***spa* sequence of GM-4:(t267)**

GAGGAAGACAACAACAAACCTGGTAAAGAAGACGGCAACAAACCTGGCAAAGAAGACAACAACAAGCCTGGTAAAGAAGACAACAAC
AAGCCTGGCAAAGAAGACGGCAACAAGCCTGGTAAAGAAGACAACAAAAAACCTGGTAAAGAAGACAACAAAAAACCTGGTA
AAGAAGACAACAAAAAACCTGGTAAAGAAGATGGCAACAAGCCTGGCAAAGAAGACAACAAAAAACCTGGT

***spa* sequence of GM-6:(nt)**

CGTTGCGGGTCTCGCGATGCATCTAGATTCAGCAGTAGTGCCGTTTGCTTTTGCAATGTCATTTACTGTATCACCAGGTTTAAC
GACATGTACTCCGTTGCCGTCTTCTTTACCAGGTTTTTTGTTGTCTTCTTTGCCAGGCTTGTTGCCATCTTCTTTACCAGGTTTTTTGTTGT
CTTCTTTACCAGGTTTTTTGTTGTCTTCTTTACCAGGTTTTTTGTTGTCTTCTTTACCAGGTTTTTGTTGTCTTCTTTACCAGGCTTGT
TGCCGTCTTCTTTGCCAGGCTTGTTGTTGTCTTCTTTACCAGGCTTGGTGTTGTCTTCTTTGCCAGGTTTGTTGCCGTCCTCTTTACCAGGT
TTGTTGTTGTCTTCCTCTTTTGGTGCTTGAGCATCGTTTAACTTTTTAGCTTCTGGTAAAATTTCTTTGCTCACCGAAGGATCGTCTTTAA
TCGGATCCCGGGCCCGTCGACTGGAGAAGCCTGCATGCAAGCTTTCCCTATAGTGAGTCGTATTAAAACTTGGCGTAATCATGGTCATAGC
TGTTTCCTGTGTGAAATTGGTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGT
GGCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCCGGAAACCTGTCGTGCCAGCTGC
ATTAATGGATCGGCCCACGCGCGGGGAGAGGCGGTTTGGCGTAATGGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTT
CCGCTGCGCGAGCGGTATCAGCTCGACTCAAAGGCGGTAATACCGGTTATCACAGGAATCAGGGGATAACGGCAGGAAAG
AACATGTGAACAAAAGCCAGCAAAAGGCCAGACCGTAAAGGGCCGCGTTGCTGGCGTTTTCATAGGCTCCGCCCCCTGACA
AGCATCCAAAAATCGACGCCTCAGTCAAGTGGGCGAACCCGACT

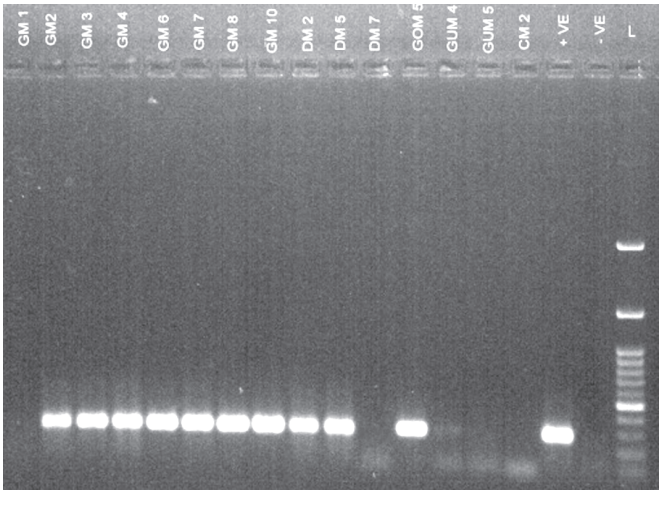


Fig. 1. Bands showing positive for *nuc* gene (272 bp).

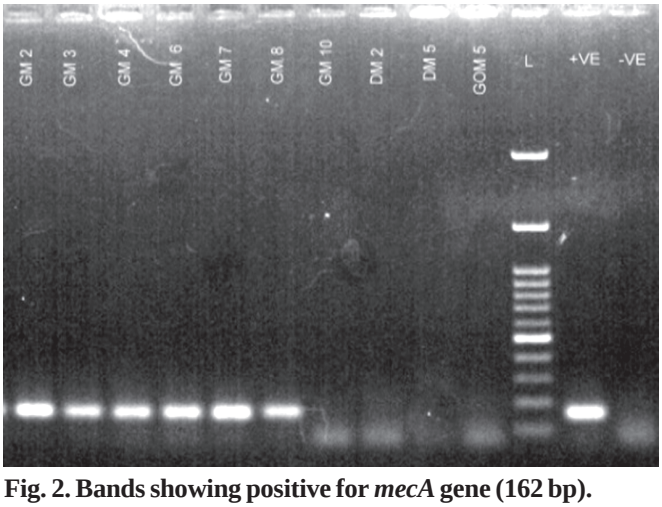


Fig. 2. Bands showing positive for *mecA* gene (162 bp).

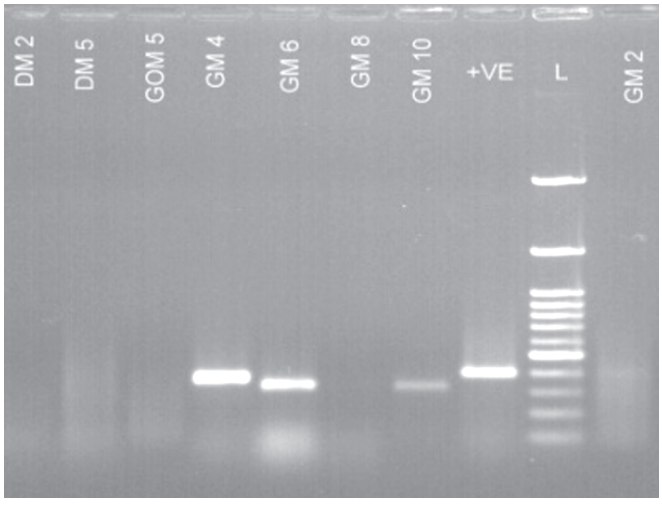


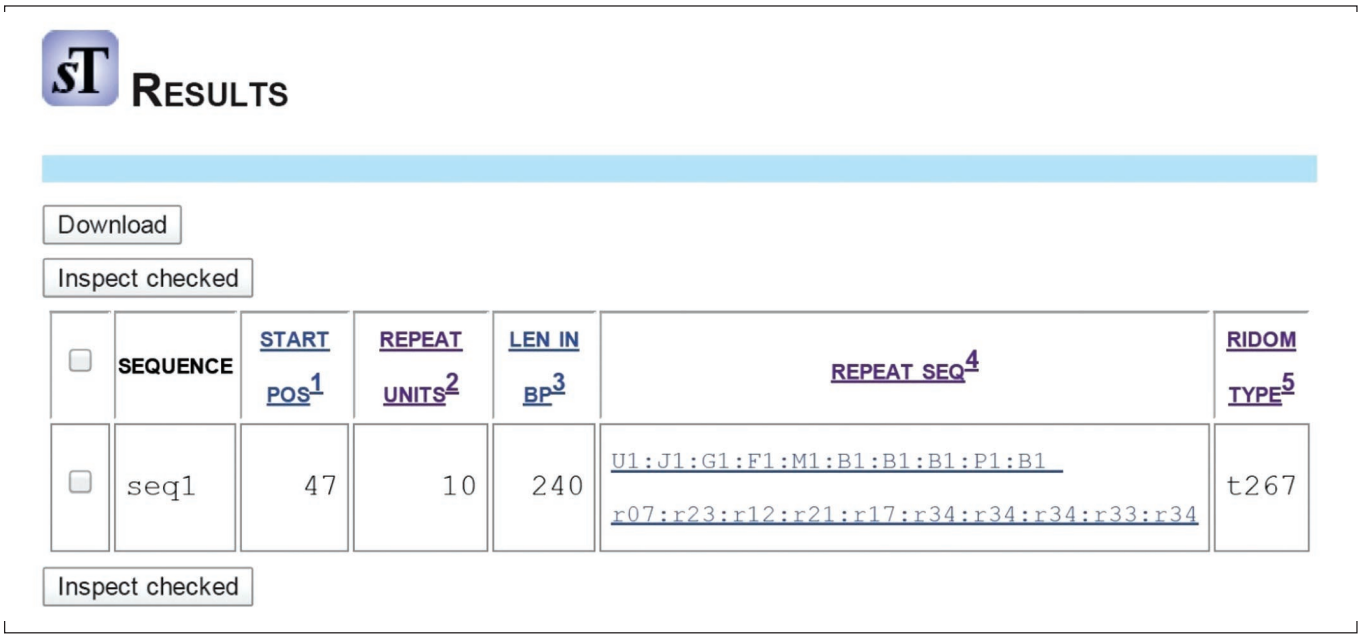
Fig. 3. Bands showing positive for *spa* gene.

aureus isolate was 6 hours for PCR. Our results were in consonance with similar study by Gosbell *et al.* (2001).

Detection of Staphylococcal Protein A (*spa*) gene and *spa* Typing

The sequence of the polymorphic VNTR in the 3' coding region of *S. aureus*-specific staphylococcal protein A (*spa*) is analyzed for *spa* typing. A unique repeat code is assigned for each new base composition of the polymorphic repeat found in a strain. The repeat succession for a given strain determines its *spa* type.

A total of 3 samples were positive for *spa* (GM-4, GM-6 and GM-10) after PCR screening (Fig. 3). They were subjected to agarose gel electrophoresis separately to obtain gel purified products. The gel purified products



RESULTS

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	SEQUENCE	START POS[1]	REPEAT UNITS[2]	LEN IN BP[3]	REPEAT SEQ[4]	RIDOM TYPE[5]
	seq1	47	10	240	U1:J1:G1:F1:M1:B1:B1:B1:P1:B1 r07:r23:r12:r21:r17:r34:r34:r34:r33:r34	t267

Inspect checked

Fig. 4. *spa* typing result of GM-4.

were sent to Scigenom (India) for sequencing. The sequences were then analyzed which had given one typeable sequence (from GM-4) and two non-type able sequences (from GM-6 and GM-10) (Fig. 4).

CONCLUSION

In the present study, the presence of MRSA strains in cattle intra-mammary infection suggests that intensive use of antibiotics may exert selection pressure for evolvement of resistant strains through dissemination and acquisition of *mecA* gene, by some mechanism still unknown.

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