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PCR-based detection of genes encoding virulence determinants in *Staphylococcus aureus* from bovine subclinical mastitis cases

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The present study was carried out to genotypically characterize *Staphylococcus aureus (S. aureus)* isolated from bovine mastitis cases. A total of 37 strains of *S. aureus* were isolated during processing of 552 milk samples from 140 cows. The *S. aureus* strains were characterized phenotypically, and were further characterized genotypically by polymerase chain reaction using oligonucleotide primers that amplified genes encoding coagulase (*coa*), clumping factor (*clf*A), thermonuclease (*nuc*), enterotoxin A (*ent*A), and the gene segments encoding the immunoglobulin G binding region and the X region of protein A gene *spa*. All of the isolates yielded an amplicon with a size of approximately 1,042 bp of the *clf*A gene. The amplification of the polymorphic *spa* gene segment encoding the immunoglobulin G binding region was observed in 34 isolates and X-region binding was detected in 26 isolates. Amplification of the *coa* gene yielded three different products in 20, 10, and 7 isolates. The amplification of the thermonuclease gene, *nuc*, was observed in 36 out of 37 isolates. All of the samples were negative for the *ent*A gene. The phenotypic and genotypic findings of the present strategies might provide an understanding of the distribution of the prevalent *S. aureus* clones among bovine mastitis isolates, and might aid in the development of steps to control *S. aureus* infections in dairy herds.

Key words: genotyping, India, *Staphylococcus aureus,* subclinical mastitis

Introduction

Staphylococcus aureus (S. aureus) is one of the common causes of subclinical mastitis worldwide, which is of economic importance to the dairy industry [13]. Raw milk is a potential source of *S. aureus* in milk and milk products, especially in the case of defective pasteurization. The main reservoir of *S. aureus* seems to be the infected quarter. Molecular epidemiological analysis of the bovine *S. aureus* population suggested that a small number of clonal types were responsible for most infections, and that strains had a broad geographic distribution [7,11,15].

S. aureus has a capacity to produce a large number of putative virulence factors [6,8,9]. Some of these factors may be of more importance than others in different diseases or at different stages of the pathogenesis of particular infections, as not all factors are produced by each strain. At present, nothing has been reported about the occurrence of these virulence factors among *S. aureus* isolates from India, and about the possible distribution of single *S. aureus* clones as causative agents of bovine mastitis at various farms. The present study was conducted to genotypically characterize *S. aureus* isolates in milk samples from cows with subclinical mastitis.

Materials and methods

Samples

A total of 552 quarter milk samples were collected from 140 cows selected randomly from 8 farms in the Vidarbha region of Central India. Samples were collected over a two month period. The samples were tested by the California mastitis test (CMT) for subclinical mastitis, and were graded as negative, trace, weak, distinct, or strongly positive [14]. Isolation of *Staphylococcus* was attempted from the CMT positive milk samples.

Phenotypic characterization

The isolates were phenotypically characterized using various cultural, morphological, and biochemical tests such as tube coagulase, urease, Tween 20 hydrolysis, and sugar fermentation [3,4]. The strains of *S. aureus* were further examined for DNase production [12].

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Genotypic characterization

Chromosomal DNA of the isolates was prepared as described by Wilson [19] with some modification. In brief, bacteria were grown in brain heart infusion broth for 24 h at 37°C. The cultures were centrifuged at 4°C at 8,000 g for 10 min. The pellet was suspended in TE buffer (200 µl) (10 mM Tris HCl + 1 mM EDTA, pH 8.0) with lysozyme (10 mg/ml) and incubated at 37°C for 2 h. The bacteria was lysed with 10% SDS and proteinase K (10 mg/ml), and was incubated at 65°C for 30 min. The denatured protein, cell wall debris, and polysaccharides were eliminated by the addition of 5 M NaCl and CTAB/NaCl (10% hexadecyl trimethyl ammonium bromide in 0.7 M NaCl) and incubated for 30 min at 65°C. DNA was purified by extraction with phenol: chloroform (1 : 1) and chloroform: isoamyl alcohol (24 : 1). DNA was precipitated with isopropanol and sodium acetate (3 M) solutions, washed in 70% ethanol, and suspended in 50 µl of TE buffer.

The virulence determinants investigated using the oligonucleotide primers included the genes encoding coagulase (*coa)*, clumping factor (*clf*A), the IgG-binding region and the X-region of protein A (*spa*), enterotoxin A (*ent*A), and thermonuclease (*nuc*). For all the genes, reaction mixtures (25 µl) included 2 µl template DNA, 10 × PCR buffer (Sigma Aldrich, USA), 25 mM $MgCl_2$, 200 µM of the four dNTPs, 10 pmol of each of the 2 primers (Bangalore Genei, India), and 1U *Taq* DNA polymerase (Sigma Aldrich, USA).

In the present study, the amplification parameters and primer sequences described by Straub *et al.* [18] were used (Table 1). The amplification of genes was carried out with thermocycler (Thermo Hybaid, USA).

Amplified products were separated by agarose gel electrophoresis (1.5% agarose containing 0.5 mg ethidium bromide in 0.5 × Tris-EDTA electrophoresis buffer) at 5 V/cm for 2 h and photographed under UV illumination.

Results

Isolation of *Staphylococcus* was attempted from CMT positive milk samples. Out of 552 milk samples collected from 140 cows on 8 farms, 501 (90.76%) samples from 134 cows were found to be CMT positive. Of these 268 milk samples, 114 cows harbored *Staphylococcus* sp. On the basis of cultural and biochemical properties, 37 isolates were identified as *S. aureus*. All 37 isolates were positive for the tube coagulase test. Others strains were identified as *S. intermedius, S. hyicus,* coagulase negative staphylococci, and *Micrococcus* (data not shown).

Amplification of the *coa* gene yielded three different products of 627, 710, and 910 bp for 20, 10, and 7 isolates from 7, 4, and 5 farms, respectively, and gene polymorphism

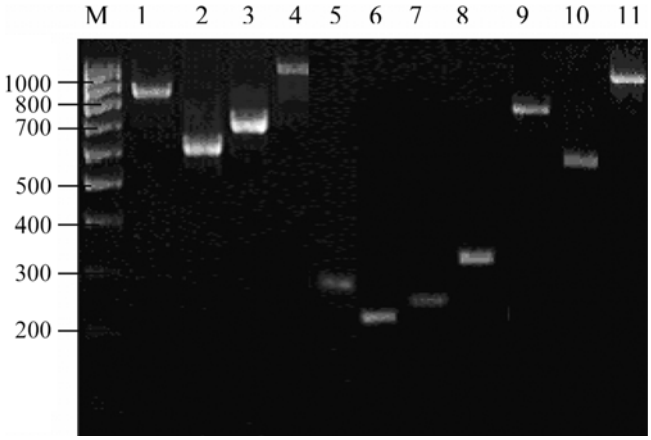

Fig. 1. Amplicons of the genes encoding Staphylococcal coagulase (*coa*), clumping factor (*clf*A), thermonuclease (*nuc*), *spa* gene X-region, and IgG-binding regions. Lane M: DNA molecular weight marker MBD 13 (Bangalore Genei, India); Lane 1-3: *coa*; Lane 4: *clf*A; Lane 5: *nuc;* Lane 6-8: *spa* X-region; Lane 9-11: *spa* IgG-binding region.

Table 1. Primers for amplification of the Staphylococcal genes

Gene	Sequence (5'-3')	PCR program*	Size of amplified products (bp)
*clf*A	forward: GGCTTCAGTGCTTGTAGG reverse: TTTTCAGGGTCAATA TAAGC	1	1042
coa	forward: ATAGAGATGCTGGTACAGG reverse: GCTTCCGATTGTTCGATGC	2	627,710,910
spa (IgG-binding)	forward: CACCTGCTGCAAATGCTGCG reverse: GGCTTGTTGTTGTCTTCCTC	1	590,810,970
spa (X-region)	forward: CAAGCACCAAAAGAGGAA reverse: CACCAGGTTTAACGACAT	3	220,253,315
nuc	forward: CGATTGATGGTGATACGGTT reverse: ACGCAAGCCTTGACGAACTAAAGC	4	279
*ent*A	forward: AAAGTCCCGATCAATTTATGGCTA reverse: GTAATTAACCGAAGGTTCTGTAGA	4	216

*1: 35 cycles 94°C-60 sec, 57°C-60 sec,72°C-60 sec; 2: 30 cycles 94°C-40 sec, 58°C-60 sec,72°C-60 sec; 3: 30 cycles 94°C-60 sec, 60°C-60 sec,72°C-60 sec; 4: 30 cycles 94°C-3 min, 58°C-30 sec,72°C-45 sec. Initial denaturation at 94°C for 5 min and final extension at 72°C for 10 min.

Table 2. Genotypic characteristics of *S. aureus* isolates from various farms of central India

Farm [No. of isolates]	Gene (bp)											
						spa						
						IgG binding region			X-region			
	*clf*A	*nuc*		*coa*								*ent*A
	(1042)	(279)	(627)	(710)	(910)	(590)	(810)	(970)	(220)	(253)	(315)	(216)
A [1]	1	1			1		1		1			-
B [4]	4	3	1	2	1	2	2		2			-
C [6]	6	6	2	2	2		6		1	3	2	-
D [11]	11	11	6	4	1	3	5	1	5	2	3	-
E [3]	3	3	1		2	2		1		1		-
F [3]	3	3	3			3					1	-
G [8]	8	8	6	2		2	1	4	1	2	1	-
H [1]	1	1	1					1		1		-
Total	37	36	20	10	7	12	15	7	10	9	7	-

was observed in isolates originating from 5 farms. All of the isolates yielded an amplicon with a size of approximately 1,042 bp of the *clf*A gene. The amplification of the gene segment encoding the IgG binding region of protein A (*spa*) revealed a size of 590, 810, and 970 bp in 12, 15, and 7 isolates from 5, 5, and 4 farms, respectively, and gene polymorphism was noted in isolates from 4 farms. The X-region binding of the *spa* gene produced an amplicon of 220, 253, and 315 bp in 10, 9, and 7 isolates, respectively. The amplification of the extracellular thermonuclease *nuc* gene produced an amplicon of 279 bp in 36 out of 37 isolates. All of the samples were found to be negative for the *ent*A gene. Amplicons specific to the *coa*, *clf*A, *nuc, spa* IgG binding, and X-region genes are shown in Fig. 1. The genotypic properties of the 37 *S. aureus* isolates are summarized in Table 2.

Discussion

S. aureus has been recognized as a pathogen in human and animal infections. In the present study, 37 *S. aureus* strains isolated from subclinical bovine mastitis cases were identified and further characterized by PCR amplification of various virulence genes encoding clumping factor and coagulase activity, and gene segments encoding the immunoglobulin G-binding region and X-region of protein A and stable thermonuclease activity. Comparable PCR-based detection studies of the virulence genes have been described by other investigators [1,15].

The *coa* and *spa* (IgG-binding region and X-region) genes investigated in the present work exhibited typical gene polymorphism. This attribute could be used for the genotypic characterization of single isolates of this species. The *spa* gene segments encoding the X-repetitive region are known to consist of a variable number of small repeats [5]. It is thought that the *spa* domain encoding the X-region may serve to extend the N-terminal IgG-binding portion of the protein through the cell wall. It was interesting to note that isolates from the same farm exhibited polymorphism among the *coa* and *spa* genes.

The ability of *S. aureus* to adhere to extracellular matrix proteins is thought to be essential for the colonization and the establishment of infections [5]. *S. aureus* possesses various adhesion genes, including *clf*A, *fnb*A, and *cna* [16]. PCR analysis of the other virulence genes revealed the *nuc* and *clf*A genes in 36 and 37 strains, respectively, of the 37 strains investigated, suggesting an important role of these elements in the pathogenecity of bovine mastitis. However, *ent*A was not present among the strains. In contrast, combined occurrence of enterotoxin genes has been described by other investigators [1,10,17,20].

In the present study, *S. aureus* isolates from cattle with bovine mastitis were found to differ in their gene patterns. Phenotypic and genotypic characterization might provide a better understanding of the distribution of the prevalent *S. aureus* clones among bovine mastitis isolates. This can aid in the investigation and control of *S. aureus* infections in dairy herds.

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