VETERINARSKI ARHIV 78 (1), 31-38, 2008

Epidemiological typing of *Staphylococcus aureus* by DNA restriction fragment length polymorphism of *coa* gene

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SANJIV, K., A. K. KATARIA, R. SHARMA, G. SINGH: Epidemiological typing of *Staphylococcus aureus* by DNA restriction fragment length polymorphism of *coa* gene. Vet. arhiv 78, 31-38, 2008.

ABSTRACT

Twenty one *Staphylococcus aureus* isolates of bovine mastitic milk origin from herds at different locations were analysed for their *coa* gene products and RFLP patterns. One of the isolates was *coa* gene deficient whereas the rest revealed polymorphism in the *coa* gene. The isolates revealed three different types of *coa* gene products (600, 680 or 850 bp) and three distinct RFLP patterns were obtained with *Alu*I digests of PCR products. Amplicons of 600 bp produced only one fragment of 300 bp (Pattern I), amplicons of 680 bp produced two fragments of 210 and 260 bp (pattern II) and amplicons of 850 bp produced 3 bands at 170, 290 and 390 bp (pattern III). It was concluded that not all the *S. aureus* isolates possessed the *coa* gene, the *coa* genotype was location-specific and this character of the isolates can be used in epidemiological investigations.

Key words: *Staphylococcus aureus,* mastitis, *coa* gene, restriction fragment length polymorphism

Introduction

Mastitis is one of the most important diseases of cattle throughout the world causing great economical losses. *Staphylococcus aureus,* the major mastitis pathogen has been shown to exhibit different phenotypic properties including variable response to antibiotics *in vitro* as well as *in vivo*.

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ISSN 0372-5480
Printed in Croatia

Though the phenotypic studies help to identify and to understand the distribution of prevalent *S. aureus* clones among dairy herds, the DNA based molecular typing approaches can be of much use for this purpose.

Coagulase production by *S. aureus* is considered by clinical microbiologists to be an important criterion for identification of *S. aureus* and different allelic forms of this enzyme has been demonstrated to exist. The *coa* gene coding for coagulase protein has also been used for the development of DNA based diagnostic assays for *S. aureus*.This gene, being highly polymorphic because of variable sequences at its 3' coding region, does not permit ubiquitous identification of all strains of *S. aureus* (SCHWARZKOPF and KARCH, 1994) but it can be used for differentiation of *S. aureus* isolates (GOH et al., 1992).

In the present investigation, 21 isolates confirmed as *S. aureus* by ribotyping were subjected to molecular typing for *coa* gene polymorphism to see the epidemiological relationship among them.

Materials and methods

S. aureus isolates and their identification. The organisms isolated from milk samples from cattle with clinical mastitis were identified as *S. aureus* (COWAN and STEEL, 1975). The tests for identification of isolates included growth in air, Gram's reaction, acid-fastness, morphology, motility, spore formation, glucose fermentation, catalase activity (QUINN et al., 1994), oxidase test (FALLER and SCHLEIFER, 1981) and oxidation-fermentation test (HUGH and LEIFSON, 1953). The tests for metabolic and biochemical reactions (Secondary tests) included coagulase production in test tubes using human plasma, sugar fermentation, haemolysis on blood agar and toxin production.

The isolates were further genetically confirmed by ribotyping (23S rRNA gene based identification) as per the method described by STRAUB et al. (1999). From cultures grown overnight DNA was isolated as per the method of NACHIMUTTU et al. (2001). Proteins, including RNA were removed from DNA by treating with DNAase free RNAase and DNA quantification was carried out by spectrophotometric measurements (SAMBROOK et al., 1989). Quantified DNA was diluted to a final concentration of 50 ng/μL in Tris-EDTA buffer. The PCR was performed using the following primers:

Primer -1:5'- ACGGAGTTACAAAGGACGAC- 3'

Primer -2:5'- AGCTCAGCCTTAACGAGTAC- 3'

The reaction mixture (total volume 30 μL) was prepared by mixing 1 μL primer-1 (10 pM/μL), 1 μL primer-2 (10 pM/μL), 0.6 μL dNTP (10 mM), 3 μL 10× buffer, 1.8 μL $MgCl_2$ (2.5 μM/μL), 0.1 μL *Taq* DNA polymerase (5 U/μL), 20 μL deionised water and 2.5 μL DNA (50 ng/μL).

The PCR products, after addition of 2 μL of trekking dye, were resolved in 1.5% agarose gels prepared in 1× Tris-EDTA buffer containing 0.5 μg/mL of ethidium bromide and 100 bp DNA ladder was used as a molecular marker. The amplification products were electrophoresed for 5 hours at 50 Volts. The gel was then visualised under U.V. transilluminator.

Coa gene amplification. The PCR was performed for *coa* gene amplification as per the method described by HOOKEY et al. (1998). The following sequences for two primers were used:

Primer - 1:5'- ATAGAGATGCTGGTACAGG-3'

Primer - 2:5'-GCTTCCGATTGTTCGATGC-3'

The PCR mixture (total volume 30 μL),) was prepared by mixing primer-1, 0.5 μL (75 pM/μL), primer-2, 0.5 μL (75 pM/ μL), 2.5 μL 10× buffer, 3 μL $MgCl_2$ (2.5 μM/μL), 1 unit of *Taq* polymerase, 0.5 μL dNTP mix (10 mM/μL), deionised water 20 μL and DNA template 2.5 μL (100 ng/μL). The PCR was performed and products were electrophoresed for 5 hours at 50 Volts in 1.5% agarose gels.

RFLP of coa gene product. Restriction fragment length polymorphism of PCR *coa* gene product digested by *Alu*I was carried out (HOOKEY et al., 1998).The PCR product (10 μL) was added with nuclease-free water (16 μL), 10× Buffer Tango (2 μL) and *Alu*I (2 μL), mixed gently and spun down for a few seconds, then incubated at 37 °C for 3 hours. The digest, after addition of 2 μL of trekking dye, was resolved in 2.75% agarose gel, prepared in 1× Tris-EDTA buffer, containing 0.5 μg/mL of ethidium bromide and 100 bp DNA ladder, was used as the molecular marker. The amplification products were electrophoresed for 5 hours at 50 Volts and the gel was then visualised under a U.V. transilluminator.

Results

Out of 21 *S. aureus* isolates, 20 isolates produced round, smooth and glistening colonies after 24 hrs incubation at 37 °C, with golden yellow pigmentation on nutrient agar, showed a strong coagulation reaction with human plasma and did not ferment raffinose, whereas, the colony of one isolate (no. 16) was white in colour, did not show coagulation and fermented raffinose. Mannitol, maltose and dextrose were fermented by all the isolates. All the 21 isolates in the present investigation were haemolytic and toxigenic. The twenty isolates produced both α- and β-toxin but isolate no. 16 did not produce β-toxin, which was exhibited on agar plates as a very narrow zone of complete haemolysis.

Table 1. *Coa* gene amplicons and RFLP patterns of *S. aureus* isolates from clinical mastitis in cattle

Serial N°	Isolate N° (Total isolates)	*coa* gene amplicon (bp)	RFLP fragment size (bp)	Patterns
1.	25, 41, 44, 46, 57, 60, 61, 63, 66, 67 (10)	600	300	I
2.	8, 10, 13, 26, 30 (5)	680	210, 260	II
3.	4, 7, 11, 18, 27 (5)	850	170, 290, 390	III

Table 2. Area location-wise *S. aureus* isolates along with their *coa* amplicons and RFLP patterns

Serial N°	Area Location (Bikaner city)	Isolate N°	*Coa* gene amplicon (bp)	RFLP pattern
1.	AL - 1 (Pareek Chowk)	4, 7	850	III
2.	AL - 2 (Rathkhana)	18, 26, 30	680, 850	II and III
3.	AL - 3 (Beechwal)	8, 10, 11, 13, 25, 27, 41, 44, 46, 57	600, 680, 850	I, II and III
4.	AL - 4 (Gangashahar)	60, 61, 63, 66, 67	600	I
5.	AL - 5 (Gandhi Colony)	16	-	-

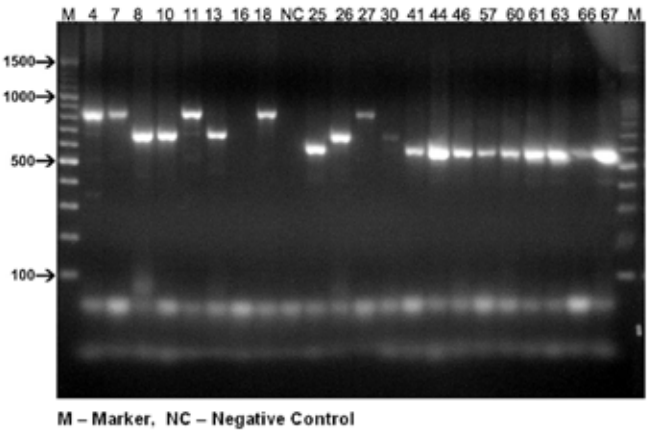

Fig. 1. *Coa* gene polymorphism in *S. aureus* obtained with specific primer

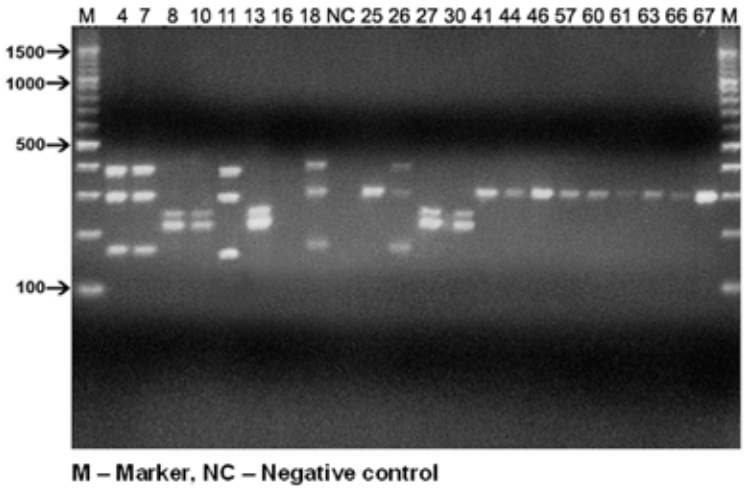

Fig. 2. RFLP patterns generated from *Alu*I digests of *coa* gene amplicons of *S. aureus*

The same one *S. aureus* isolate did not give any amplification product but the remaining 20 isolates produced one of the three types (600, 680 or 850 bp) of *coa* gene products (Table 1, Fig. 1). When the isolates from different area locations were analysed for the presence of a particular *coa* gene product (Table 2), it was observed that isolates from area location AL-1 had one type of product (850 bp), isolates from AL-2 had 2 gene products (680 bp, 850 bp), isolates of AL-3 had gene product of all sizes (600, 680 and 850 bp) and isolates of location AL-4 had single sized gene product (600 bp). The data revealed an epidemiological difference on the basis of the *coa* whole gene product.

The *coa* gene PCR product of all the 20 isolates were digested with *Alu*I and RFLP patterns were obtained (Table 1). In the investigation 3 distinct RFLP patterns were observed (Fig. 2). Amplicons from ten of the isolates produced only one fragment of 300 bp (pattern I), 5 produced two fragments of 210 and 260 bp (pattern II) and the remaining 5 produced a RFLP pattern having 3 bands at 170, 290 and 390 bp (pattern III).

Discussion

In the present investigation one isolate did not give any *coa* gene amplification product indicating the absence of this gene in the isolate. This isolate also did not produce coagulase as observed phenotypically in the test tube and was considered *coa* gene deficient. Similarly KOBAYASHI et al. (1995) did not show *coa* gene amplification

in one of the 240 *S. aureus* strains, though it was phenotypically identified as coagulase producer.

The other 20 isolates produced one of three types of *coa* gene products of 600, 680 or 850 bp size, whereas HOOKEY et al. (1998), using the same probe, obtained four different amplification products of 547, 603, 660 or 875 bp. SALASIA et al. (2004) also carried out *coa* gene typing using similar primer, with *S. aureus* isolated from bovine sub-clinical mastitis and found two amplicons of 600 and 850 bp in Central Java and 5 amplicons of 510, 600, 680, 740 and 850 bp sizes in Germany. The variability in the amplification products reflects the variation in the *coa* gene lengths of *S. aureus* isolates. The presence of only three types of bands in the present study indicated that there were lesser variations in the tandem repeats of 81bp as comparison to the repeats in the isolates of the above two scientists, as amplicons comparable to 510, 547 or 740 bp were not recorded in our study. KAIDA et al. (1987) described that 3' end of *coa* gene contains a series of 81bp tandem repeats, the number of which differs between strains. GULER et al. (2005) also obtained 4 types of coagulase gene PCR products for *S. aureus* isolated from bovine clinical mastitis.

A difference in the amplicon sizes was obtained between our study and that of HOOKEY et al. (1998) and SALASIA et al. (2004) by use of the same primers. A size difference of 10-20 base pairs for PCR products of *coa* gene of *S. aureus* has been suggested by HOOKEY et al. (1998).

The data in our study revealed an epidemiological difference in the genotypes of the isolates on the basis of *coa* whole gene product. LAWRENCE et al. (1996) used results of coagulase gene polymorphism and detected methicillin resistant *S. aureus* in nasal swabs and suggested that this method could be particularly useful in outbreaks where rapid control of MRSA spread is required.

The digestion of different *coa* gene PCR products with *Alu*I, generated distinct RFLP patterns. It is suggested that 600 bp amplicon of 10 isolates showing RFLP pattern I was cut into two equal halves of 300 bp by *Alu*I. A similar pattern of digestion was also obtained by HOOKEY et al. (1998) who recorded single fragments when *S. aureus coa* gene amplicon of 600 bp was digested with *Alu*I. The pattern II with two fragments from 680 bp amplicon suggested digestion at two restriction sites giving rise to generation of two fragments of the same size (210 bp) and one of different size (260 bp).

The RFLP pattern III suggested the presence of two restriction sites within amplicon of 850 bp producing 3 fragments of 170, 290 and 390 bp. One of the fragments in pattern III (290 bp) was similar to the fragment of RFLP pattern I indicating similar restriction site in these isolates. This pattern of obtaining similar fragments can be correlated to their clonal relationships. KOBAYASHI et al. (1995) carried out similar studies on *coa* gene products and RFLP patterns using *Alu*I for *S. aureus* isolates and demonstrated the utility

of RFLP typing in epidemiological investigations of *S. aureus* infection in hospitals. GOH et al. (1992) used coagulase RFLP and successfully traced the source of an outbreak of methicillin resistant *S. aureus* infection.

The isolates from different locations were analysed for their *coa* gene product and RFLP patterns and it was observed that both the isolates from AL-1 and all the 5 isolates from AL-4 were similar. Likewise the single isolate from AL-5 was altogether different from the isolates obtained from all other locations. This finding indicated that the genotype was also location-specific. The isolates from AL-2 as well as from AL-3 were not similar genotypically. The reason could have been the fact that at these two locations there were regular influx and outflow of animals, bringing different organisms from other locations.

In the present investigation different *coa* gene products and their restriction fragment length polymorphism for *S. aureus* isolates indicated great genotypic variability among the organisms. It was concluded that RFLP can be used to study the genotype of the organisms and to trace the source of their origin for epidemiological purposes in the event of disease outbreaks.

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Received: 28 August 2006
Accepted: 28 December 2007

SANJIV, K., A. K. KATARIA, R. SHARMA, G. SINGH: Epidemiološka tipizacija bakterije *Staphylococcus aureus* na osnovi DNA polimorfizma duljine restrikcijskoga fragmenta gena *coa*. Vet. arhiv 78, 31-38, 2008.

SAŽETAK

Dvadeset i jedan izolat bakterije *Staphylococcus aureus* iz mlijeka krava s upalom vimena iz različitih stada bio je analiziran na proizvode njezina gena *coa* i polimorfizam duljine restrikcijskih fragmenata (PDRF). U jednom izolatu nije bio dokazan gen *coa* dok je u ostalim ustanovljen polimorfizam u tom genu. Izolati su pokazali tri različita tipa proizvoda gena *coa* (600, 680 ili 850 bp), a cijepanjem PCR proizvoda enzimom AluI dobivena su tri različita uzorka PDRF. Amplikoni od 600 bp imali su samo jedan fragment od 300 bp (uzorak I). Amplikoni od 680 bp pocijepali su se na dva fragmenta, jedan od 210, a drugi od 260 bp (uzorak II). Amplikoni od 850 bp pocijepali su se na tri fragmenta: 170, 290 i 390 bp (uzorak III). Zaključuje se da svi izolati vrste *Staphylococcus aureus* ne posjeduju gen coa, te da je genotip *coa* bio specifičan po svojoj lokaciji i da se ta značajka izolata može rabiti u epidemiološkim istraživanjima.

Ključne riječi: *Staphylococcus aureus*, mastitis, gen *coa*, polimorfizam duljine restrikcijskih fragmenata