Vol 19 No 2, p 129-134

POLYMORPHISM IN *COA* AND *SPA* VIRULENCE GENES IN *STAPHYLOCOCCUS AUREUS* OF CAMEL SKIN ORIGIN

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ABSTRACT

The present study was undertaken with a view to identify *S. aureus* organisms isolated from camel skin wounds by ribotyping and to study polymorphism in their two virulence genes viz. *coa* and *spa*, responsible for production of coagulase and protein A, respectively. All the 15 isolates in the study were ribotyped targeting 23S rRNA gene using species specific primers wherein a species specific amplicon of 1250 bp was produced. The polymerase chain reaction targeting *coa* gene produced only single amplicon in each isolate but of variable sizes. The frequency of amplicon of 600 bp was highest followed by amplicons of 710, 760 and 850 bp. The PCR amplification of X-region of *spa* gene produced amplicons of seven different sizes viz. 160, 180, 200, 260, 280, 300 and 500 bp with calculated number of 5, 6, 7, 9, 10, 11 and 19 repeats, respectively. Out of 15 isolates 14 produced only single amplicon whereas one isolate produced two amplicons of 200 and 500 bp size. In the present study most of the isolates possessed seven repeats of 24 bp in variable X-region of *spa* gene. The present investigation revealed presence of both pathogenic and nonpathogenic strains of *S. aureus* with polymorphism in both the virulence genes.

Key words: Camel, polymorphism, *S. aureus*, virulence genes

The literature regarding microbiology of skin wounds and abscesses in camel is scarce. Different workers have isolated *Staphylococcus aureus*, a major cause of wounds and abscesses in camel, from skin lesions during past but information remains incomplete as far as strain identification and strain variation is concerned since this organism shows variations in phenotypic expressions (Boerlin *et al*, 2003). The identification of strains is important to confirm the epidemiological relationships among them (Aarestrup *et al*, 1994) and for appropriate antibiotic therapy (Marcos *et al*, 1999). The molecular typing approaches have been reported to be of greatest advantage in identifying and monitoring the local and international spread of *S. aureus* strains (Diep *et al*, 2003).

Virulence factors like coaguase and protein A of *S. aureus* encoded by *coa* and *spa* gene, respectively have been demonstrated to be directly related with pathogenesis and severity of infection (Sharma *et al*, 2000; Akineden *et al*, 2001). Both of the genes are highly polymorphic and can provide important information in regards to strain variations. Since there can be involvement of various strains of *S. aureus* in the causation of wounds and abscesses it is imperative that the strains involved be identified with greater accuracy.

Since there is paucity of literature in regards to genetic studies on *S. aureus* isolates of camel origin, the present investigation was undertaken with a view to genotype these isolates for their identification and to see the polymorphism in their *coa* and *spa* genes.

Materials and Methods

Sampling, isolation and identification of Staphylococcus aureus

The pus samples were collected from camels with skin wounds which were being used mostly for load carrying. All the animals were adult and belonged to both sexes. The pus samples were collected aseptically with sterile absorbent swabs soaked in nutrient broth from different sites on animal body. After collection, the samples were immediately taken to laboratory over ice for further processing.

The samples were inoculated on nutrient agar plates and then processed for isolation and identification of *S. aureus* based on the primary and secondary test characteristics using standard procedures (Cowan and Steel, 1975; Quinn *et al*, 1994).

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Genotypic confirmation of organisms by ribotyping

The DNA was isolated as per the method described by Nachimuttu *et al* (2001) with some modification. In brief, the overnight grown bacterial culture was pelleted, washed twice with PBS and resuspended in 1 ml of Tris-EDTA (TE) solution. One hundred µl of lysozyme solution (conc. 3 mg/ml) was added and mixture was then incubated at 37°C in water bath for 15 min. One hundred µl of 10% SDS solution and 2 µl of proteinase K (10 mg/ml) solution was added, incubated at 60°C in water bath for 1 h with gentle mixing at 10 min intervals, then 0.75 ml of DNA extraction buffer was added and further incubated for 30 min at 60°C in water bath. An amount of 0.5 ml of chloroform: isoamyl alcohol mixture (24:1) was added to the DNA preparation and mixed gently for about 10-15 min, mixture centrifuged at 15000 rpm for 15 min at 20°C and upper aqueous phase containing DNA was transferred to another tube.To this mixture 0.5 ml of cold isopropanol was added and the tube was replaced on ice for 15 min and then centrifuged at 15000 rpm for 10 min at 20°C. The supernatant was discarded and the pellet was dissolved in 0.5 ml of cold 70% ethanol and centrifuged at 10000 rpm for 10 min at 20°C. Supernatant was discarded and the tubes were inverted on a filter paper for 5 min and then left at room temperature for overnight to allow evaporation of alcohol. Next morning the pellet was redissolved in 50 µl of TE buffer and left for 24 h for dissolution of the pellet.

After quantification (Sambrook *et al,* 1989) the DNA was diluted to a final concentration of 25 ng/µl in TE buffer. The ribotyping based on 23S rRNA gene was carried out as per the method described by Straub *et al* (1999) using Primer-1 (5′-ACGGAGTTACAAAGGACGAC-3′) and Primer-2 (5′-AGCTCAGCCTTAACGAGTAC-3′).

A total volume of 30 µl reaction mixture for PCR was prepared by mixing 1 µl primer-1 (10 pM/µl), 1 µl primer-2 (10 pM/µl), 2.0 µl dNTP (10 mM), 3.5 µl 10x Taq buffer A containing 15mM Mgcl2, 1 unit (0.33 µl) Taq DNA polymerase (3 U/µl), 20.00 µl deionised water and 2.5 µl DNA (25 ng/µl). The denaturation, primer annealing and primer extension was carried out at 94°C, 55°C and at 72°C, respectively, in each cycle and the time given for denaturation, primer annealing and primer extension for cycle 1 was 300 sec, 30 sec and 75 sec, respectively; for cycle 2-37 it was 40 sec, 60 sec and 75 sec, respectively; and for cycle 38 it was 60 sec, 60 sec and 180 sec, respectively.

The PCR products, after addition of 2 µl of trekking dye were resolved in 1.2 % agarose gels prepared in 0.5 x TBE buffer containing 0.5 µg/ml of ethidium bromide and 500 bp DNA ladder was used as molecular marker. The amplification products were electrophoresed for 1h 30 min at 100 V. The gel was then visualized under U.V. transilluminator.

coa gene amplification

The amplification of *coa* gene was carried out as per method described by Hookey *et al* (1998) using primers with sequence of 5′-ATAGAGATGCTGGTACAGG-3′ (primer-1) and 5′-GCTTCCGATTGTTCGATGC-3′ (primer-2). A total volume of 30 µl PCR mixture was prepared by mixing primer-1, 0.5 µl (75 pmol/µl), primer-2, 0.5 µl (75 pmol/µl), 3.5 µl 10x Taq buffer A containing 15 mM $Mgcl_2$, 1 unit of Taq polymerase (3 U/µl), 2.0 µl dNTP mix (10 mM/µl), deionised water 21.0 µl and DNA template 2.5 µl (25 ng/µl). The PCR was performed in 30 cycles. The denaturation, primer annealing and primer extension was carried out at 94°C, 57°C and at 70°C, respectively for cycles 1-29 and at 94°C, 57°C and 72°C, respectively for cycle 30. The time given for denaturation, primer annealing and primer extension for cycle 1 was 45, 15 and 15 sec, respectively; for cycles 2-29 it was 20, 15 and 15 sec, respectively; and for cycle 30 it was 20, 15 and 120 sec, respectively. The PCR products were resolved in 1.2% agarose gels in the same manner as in ribotyping and 100 bp ladder was used as molecular marker.

spa gene amplification

The amplification of *spa* gene was done as per method described by Frenay *et al* (1996) using primers with sequence of 5′-CAAGCACCAAAAGAGGAA-3′(primer-1) and 5′- CACCAGGTTTAACGACAT-3′ (primer-2). The PCR mixture (total volume 30 µl), was prepared by mixing primer-1, 1.0 µl (75 pmol/µl), primer-2, 1.0 µl (75 pmol/µl), 3.5 µl 10x Taq buffer A containing 15mM Mgcl2, 1 unit of Taq polymerase (3 U/µl), 2.0 µl dNTP mix (10 mM/µl), deionised water 20.0 µl and DNA template 2.5 µl (25 ng/µl). The PCR was performed in 30 cycles. The denaturation, primer annealing and primer extension was carried out at 94°C, 55°C and at 72°C, respectively for 60 seconds. The PCR products were resolved in 1.2 % agarose gels in the same manner as that for *coa* amplicons.

Result and Discussion

The camel is an important animal species of desert ecosystem and is one of the main means of desert economy as it is used for various purposes,

the camel dairying being the latest enterprising industry. *Staphylococcus aureus* is the most important pathogen causing abscesses and wounds in camel. The identification and characterization of *S. aureus* is an important criterion in epidemiological investigations in order to identify the spread and transmission of various strains among the farms, among the various animal species and transmission to human as well. In the present study *S. aureus* organisms isolated from camel skin wounds were first identified by standard biochemical methods and then were confirmed by ribotyping using species specific primers. Further, these organisms were characterized for genes responsible for coagulase production (*coa*) and protein A production (*spa*).

Staphylococcus aureus is identified by its phenotypic characteristics among which colony pigmentation, fermentation of maltose and mannitol and production of coagulase serve as the important criteria among other biochemical reactions. However, because of variable reactions in phenotypic properties there has always been a requirement to develop DNA based technology which can identify *S. aureus* strains with certainty. Though various PCR based detection systems have been developed for identification of *S. aureus* targeting some specific genes but have not been found foolproof as these specific genes are not present in all *S. aureus* strains. In an improvement over the DNA based methods Straub *et al* (1999) developed a 23S rRNA gene based system which essentially identifies *S. aureus*. Similar studies were conducted by Stephan *et al* (2001); Salasia *et al* (2004); Sanjiv *et al* (2008); Momtaz *et al* (2010); Upadhyay *et al* (2010) for genotypic confirmation of *S. aureus* strains isolated from bovine mastitis. In the present investigation all the 15 isolates were subjected to PCR amplification targeting 23S rRNA gene using species specific primers. With the PCR a species specific amplicon of 1250 bp was produced by all the isolates (Fig 1) confirming their identification as *S. aureus*.

Coagulase gene has been demonstrated to be highly polymorphic and on PCR amplification different amplicons are obtained with different isolates. The *coa* gene at its 3′ end contains a series of 81 bp repeats in tandem which may vary in numbers leading to polymorphism (Kaida *et al*, 1987; Phonimbaeng *et al*, 1990). The scientists have made use of this property of coagulase gene in differentiation of *S. aureus* strains for various purposes.

In the present study all the *S. aureus* isolates from camel wounds produced only single *coa* amplicon in each isolate but of variable sizes (Fig 2; Table 1). Amplicons of 600 bp, 710 bp, 760 bp and 850 bp were produced by six, four, three and two isolates, respectively. When the number of repeats were calculated, the isolates with 600, 710, 760 and 850 bp amplicons were found to possess seven, eight, nine and ten repeats of 81bp, respectively. The present observations are in full agreement to those of Saei *et al* (2009) who carried out molecular typing of *S. aureus* isolated from bovine mastitis based on polymorphism of coagulase gene. They demonstrated that several variants of coagulase gene were present but only few of them were predominant suggesting contagious transmission, a common source or host adaptation of subset of population of *S. aureus* strains. Their study also indicated that genetic heterogeneity among isolates within and among herds in different regions. Similarly da Silva and da Silva (2005) and Aslantas *et al* (2007) also reported presence of many variants of coagulase genotypes in regions of their study but they indicated that only few genotypes predominated.

The amplicon sizes obtained in the present study are much similar to that obtained by Salasia *et al* (2004) who used similar primers as in the present study for typing of *S. aureus* from bovine sub-clinical mastitis. They recorded presence of 510, 600, 680, 740 and 850 bp amplicons in the isolates from Germany.

The amplicon of sizes 510, 600, 680, 710 and 850 bp were also obtained in mastitic cattle and

Table 1. *coa* gene polymorphism in *S. aureus* isolates from camel wounds.

S. No.	No. of isolates	*coa* gene amplicon size (bp)	No. of repeats
1.	6	600	7
2.	4	710	8
3.	3	760	9
4.	2	850	10

Table 2. *spa* gene (x-region) polymorphism in *S. aureus* isolates from camel wounds.

S. No.	No. of isolates	*spa* gene amplicon size (bp)	No. of repeats
1.	1	160	5
2.	2	180	6
3.	6	200	7
4.	3	260	9
5.	1	280	10
6.	1	300	11
7.	1	200, 500	7, 19

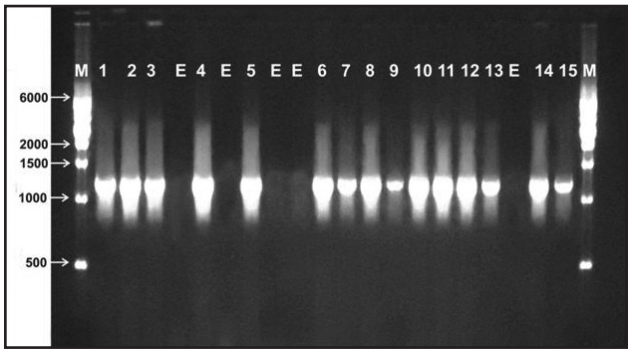

Fig 1. 23 S rRNA based ribotyping of *S. aureus* isolates from camel skin wounds.
M=Molecular marker (500 bp DNA ladder)
1-15=Isolate numbers
E=Lane without *S. aureus* DNA.

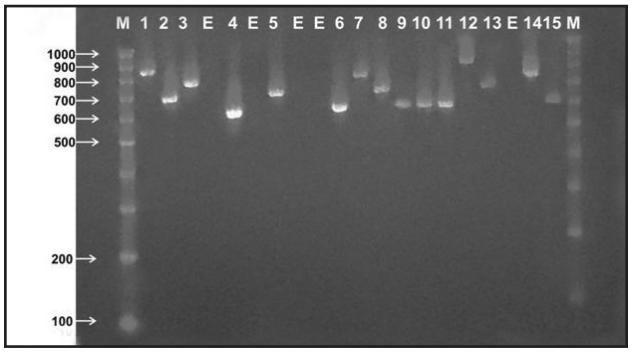

Fig 2. Coa gene polymorphism in *S. aureus* isolates from camel skin wounds.
M=Molecular marker (500 bp DNA ladder)
1-15=Isolate numbers
E=Lane without *S. aureus* DNA.

goat isolates from the same area (Sanjiv *et al,* 2008) ; Upadhyay *et al,* 2010; Khichar, 2011). The comparison of the results from those of the above predecessors from the same laboratory showed that three of the amplicons (600, 710 and 850bp) were common to isolates from cattle, goat and camel but one amplicon of 760 bp was detected in camel isolates only and amplion of 680 bp was missing from these isolates. The reason for the polymorphism in the same area in the isolates seems to be because of deletion or insertion mutations leading to removal or addition of several nucleotides consequently changing the *coa* gene size (El-Jakee *et al,* 2010).

With all the isolates only single coagulase gene amplicon was obtained in the present study, which is in accordance to observations of most of the researchers. However, two bands of *coa* gene amplicons have also been reported (da Silva and da Silva, 2005; Aslantas *et al,* 2007). Similarly, Karahan and Cetinkaya (2007) also recorded 16.1% of the isolates to yield two bands of *coa* PCR products and

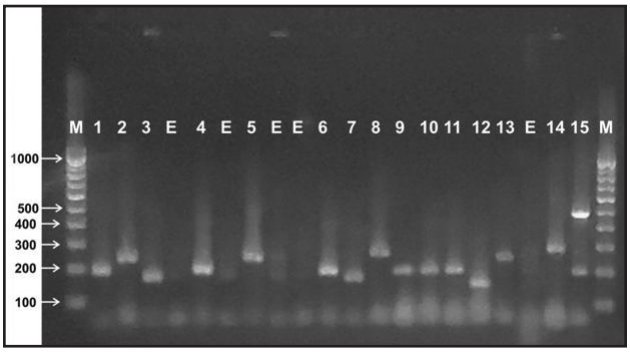

Fig 3. Spa gene polymorphism in *S. aureus* isolates from camel skin wounds.
M=Molecular marker (500 bp DNA ladder)
1-15=Isolate numbers
E=Lane without *S. aureus* DNA.

they suggested that the detection of double bands may be due to involvement of milking personnel as these isolates were reported previously from humans only.

In the present study the range of amplicon sizes was narrower i.e. between 600 and 850 bp which is similar to that in other studies viz. 740 to 990 bp (Annemuller *et al*, 1999); 620 to 809 bp (Moon *et al*, 2006); 400 to 900 bp (Coelho *et al,* 2009); 600 to 850 bp (Sanjiv *et al,* 2008); 730 to 970 bp (Momtaz *et al,* 2010); 627 to 910 bp (Salem-Bekhit *et al,* 2010); 600 to 850 bp (Upadhyay *et al,* 2010). However, a wide range of *coa* PCR amplicons have been reported by other workers viz. 579 to 1442 bp (da Silva and da Silva, 2005); 500 to 1400 bp (Karahan and Cetinkaya, 2007) and 484 to 1080 bp (da Silva *et al,* 2006).

Protein A is one of the important virulence factors of *S. aureus*. It is an antiphagocytic protein attached to cell wall with its C-terminal end, the amino terminal end being free outside and binds with Fc- region of IgG. The protein A is encoded by *spa* gene having two distinct regions. The X-region contains a varying number of small repeat units of 21-27 bp with most repeat units consisting of 24 bp (Frenay *et al,* 1996) and is highly polymorphic. The X-region is characterised by variable number of small repeats (between 3 and 15). This property of polymorphism in X-region of *spa* gene has been utilized for differentiation among *S. aureus* strains.

In the present investigation the amplification of X-region produced amplicons of seven different sizes viz. 160, 180, 200, 260, 280, 300 and 500 bp with calculated number of 5, 6, 7, 9, 10, 11 and 19 repeats, respectively (Fig 3; Table 2). The most frequent repeats observed were seven which were observed in six of the isolates. Out of 15 isolates 14 produced only

single amplicon whereas one isolate produced two amplicons of 200 and 500 bp size.

The present study is in conformity to the results of Lange *et al* (1999) who also recorded seven different amplicons of almost similar sizes and found repeat units between 5 to 12. However, six repeating units were observed most frequently by them. However, *S. aureus* without *spa* gene have also been reported by Shakeri *et al* (2010). Similarly *spa*-nontypable strain of *S. aureus* were detected by Kalorey *et al* (2007); Baum *et al* (2009); Momtaz *et al* (2010) and Salem-Bekhit *et al* (2010). They suggested that in these strains either *spa* mutation occurred or *spa* was absent.

In the present study most of the isolates possessed seven repeats of 24 bp in variable X-region of *spa* gene. However, a variable number of repeats have been reported by many workers in *S. aureus* isolates from various sources viz. Annemuller *et al,* 1999 (2-4), Stephan *et al,* 2001 (2-4), Salasia *et al,* 2004 (10-12), Reinoso *et al,* 2008 (9-10) and Bystron *et al,* 2009 (8-12).

In a study to discriminate the *S. aureus* strains on the basis of protein A Freney *et al* (1994) found that most epidemic MRSA strains harboured more than seven repeats while nonepidemic MRSA strains contains seven or fewer repeats. They discussed that a longer X-region results in a better exposition of the Fc binding region of protein A thereby facilitating colonisation on both surfaces and contributing to the epidemic phenotypes. Considering the above fact the present investigation revealed both pathogenic and non pathogenic strains.

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