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DETECTION OF GENETIC VARIATIONS AMONG *Staphylococcus aureus* ASSOCIATED WITH CAMEL SKIN WOUNDS ON THE BASIS OF *spa* AND *coa* GENE

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ABSTRACT

The present study was carried out to determine genetic variations among 26 isolates of *Staphylococcus aureus* obtained from camel skin wounds. The variations were detected by polymorphisms pattern of protein A (*spa*) and coagulase (*coa*) gene including RFLP of *coa* gene. Twenty five isolates produced seven different *spa* types (amplicon size varied between 160 and 300 bp) while one isolate did not show amplification of the gene. Of the 25 *spa* gene positive isolates, 24 isolates produced only single amplicon whereas, one isolate produced two amplicons of 260 bp and 300 bp size. All the isolates produced single *coa* gene amplicon of variable size *viz*. 510 bp, 600 bp, 680 bp, 710 bp and 760 bp size. Six RFLP patterns were detected after digestion with *Alu*I restriction endonuclease enzyme. Among different RFLP patterns, PI and PIII were found genetically closer while PVI was most distant.

Key words: Camel, *coa* gene, genetic variability, *spa* gene, *Staphylococcus aureus*

Staphylococcus aureus is the most important pathogen causing abscesses, wounds and other skin lesions such as boils, styes and furuncles in camel (Qureshi *et al,* 2002; Rathore *et al,* 2012; Lakshmi, 2015). The organism shows variations in phenotypic properties, virulence and response to antibiotic treatment. Hence, there is need to study this organism in regard to genetic variations on the basis of virulence associated genes.

Protein A is an immune evasion protein secreted by *S. aureus* and is encoded by *spa* gene. It is considered as one of the important virulence factors in the development and severity of infection (Akineden *et al,* 2001). Several studies have reported the genetic diversity in *spa* gene and also related the number of nucleotide repeats to the pathogenicity of the organisms (Karahan *et al,* 2011; Rathore *et al,* 2012). Variations among *S. aureus* strains on the basis of *spa* gene come from the differences in the repetitive variable number of 24 bp repeats in x-region of the gene and this property is being used as a molecular tool in studying the genetic diversity among the strains of *S. aureus* for epidemiological tracing of source of infection and comparing the differences in virulent phenotypes (Bhati *et al,* 2016).

Coagulase is an extracellular protein established with significant role in virulence, encoded by *coa* gene that possesses polymorphic repeat region comprising of 81 bp tandem short sequence repeats (SSRs). Thus profiling of *coa* gene and its restriction fragment length polymorphism (RFLP) can be used to measure relatedness or variations among *S. aureus* isolates (Ishino *et al,* 2007; Coelho *et al,* 2009; Saei *et al,* 2009). The PCR-RFLP of *coa* gene is a rapid, simple and efficient method for typing strains, tracing the source and transmission route of *S. aureus* infection helping to prevent and control infections (Roodmajani *et al,* 2014). The characterisation of *S. aureus* on the basis of *spa* and *coa* gene can be considered a simple and accurate method to detect molecular variations of *S. aureus* isolates (Rathore *et al,* 2012; Bhati *et al,* 2014; 2016). In the preset study, *S. aureus* isolates obtained from camel skin wounds were subjected to polymerase chain reactions taking *spa* and *coa* as target genes to see variations among them.

Materials and Methods

Sample

Forty one samples were collected from camels out of which 26 genotypically confirmed isolates

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were included in present study. All the isolates were confirmed by 23S rRNA gene ribotyping (Straub *et al,* 1999).

Spa gene amplification

The *spa* gene amplification was carried out as per method described by Frenay *et al* (1996) with some modifications. The forward and reverse primers used were 5′-CAAGCACCAAAAGAGGAA-3′ and 5′-CACCAGGTTTAACGACAT-3′, respectively. The PCR mixture (25 µl), was prepared by mixing F-primer, 1.0 µl (75 pmol/µl), R-primer, 1.0 µl (75 pmol/µl), 5 µl 10× Taq buffer A containing 15 mM $MgCl_2$, 1 unit of Taq polymerase (5 U/µl), 0.5 µl dNTP mix (10 mM/µl), deionised water 11.8 µl and DNA template 3.0 µl (25 ng/µl). The PCR was performed in 35 cycles. The denaturation, primer annealing and primer extension was carried out at 94, 55 and at 70°C, respectively for 60 s for cycles 1 to 34. The final extension was carried out at 72°C for 5 min for cycle 35. The PCR products were resolved in 1.2 % agarose gels with 50 bp molecular marker as ladder (Fig 1).

Coa gene amplification

Amplification of *coa* gene was carried out as described by Hookey *et al* (1998) using primers with sequence of 5′-ATAGAGATGCTGGTACAGG-3′ (Forward) and 5′-GCTTCCGATTGTTCGATGC-3′ (Reverse). The PCR mixture (25 µl), was prepared by mixing forward primer, 1.0 µl (10 pmol/µl), reverse primer, 1.0 µl (10 pmol/µl), 5 µl 10× Taq buffer A containing 15 mM $Mgcl_2$, 1 unit of Taq DNA polymerase (5 U/µl), 0.5 µl dNTP mix (10 mM/µl), deionised water 11.8 µl and DNA template 3.0 µl (25 ng/µl). The PCR was performed in 30 cycles. The denaturation, primer annealing and primer extension was carried out at 94, 57 and at 70°C, respectively, for cycles 1–29 and at 94, 57 and 72°C, respectively, for cycle 30. The time given for denaturation, primer annealing and primer extension was 45, 15 and 15 s for cycle 1; 20, 15 and 15 s for cycles 2 to 29; and 20, 15 and 120 s for cycle 30, respectively. The PCR products were resolved in 1.2% agarose gels with 100 bp molecular marker as ladder (Fig 2).

Restriction fragment length polymorphism (RFLP) of coa gene

The RFLP of *coa* gene was carried out with *Alu*I restriction endonuclease enzyme as described by (Hookey *et al,* 1998). The PCR product (10 µl) was added with nuclease free water (5 µl), 10× Buffer Tango (2 µl) and *Alu*I (2 units, conc. of stock enzyme was 5 U/µl), was mixed gently and incubated at 37°C for 3h. The digests were resolved in 2% MetaPhor agarose gels with 50 bp and 100 bp molecular marker (Fig 3).

Cluster Analysis

To find the genetic relatedness, cluster analysis was carried out by Dice method and cluster as mentioned in Fig 4 formed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method (http://insilico.ehu.es/dice_upgma/ online software support.

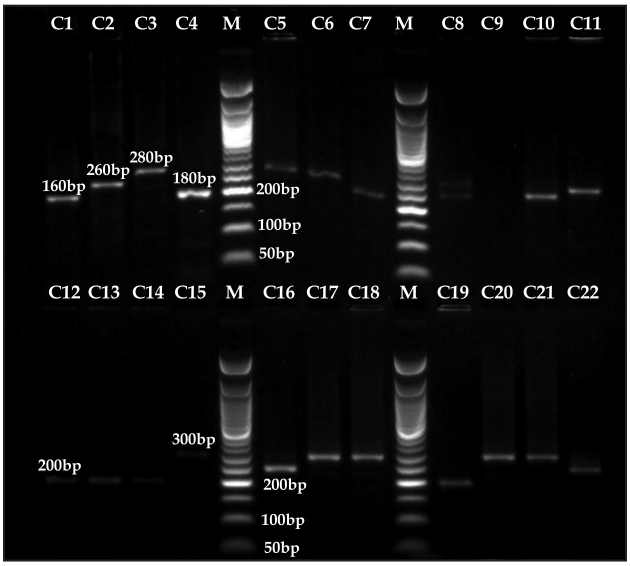

Fig 1. *Spa* gene amplicfication in *S. aureus* isolates from camel skin wounds C1 to C22, C9–*spa* gene deficient isolate C19–Isolate showing 2 amplicon of *spa* gene, M–50 bp DNA ladder.

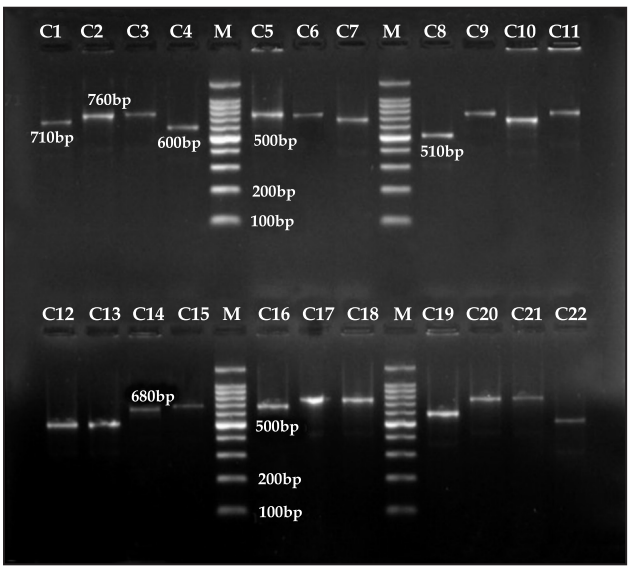

Fig 2. *Coa* gene amplicfication in *S. aureus* isolates from camel skin wounds C1 to C22, M–100 bp DNA ladder.

Table 1. *Spa* gene variations in *S. aureus* isolates from camel skin wound.

S.No.	Isolates number	Total isolates (%)	Size of *spa* amplicon (bp)	Total number of repeats
1.	C1	1 (3.84)	160	5
2.	C4	1 (38.46)	180	6
3.	C12, C13, C14, C19	4 (15.38)	200	6
4.	C2, C6, C7, C10, C16, C24	6 (23.07)	260	9
5.	C3, C5, C11, C20, C22	5 (19.23)	280	10
6.	C15, C17, C18, C21, C23, C25, C26	7 (26.92)	300	11
7.	C8	1(38.46%)	260, 300	9 and 11
8.	C9	1(38.46%)	Absent	–

Results and Discussion

The camel is comparatively less prone to many of the diseases, however, the skin infections including wounds and abscesses are a problem in camel resulting into its reduced working efficiency (Wernery *et al,* 2014). The most common organism in skin wounds has been found to be *S. aureus*. The present investigation was carried out with a view to find out genetic variations among *S. aureus* especially, in relation to *spa* and *coa* virulence genes. All the isolates in the study were confirmed by 23S rRNA based ribotyping which produced 1250 bp species specific amplicons.

Variations in spa gene

The x-region of *spa* gene was amplified in 25 isolates while one isolates (C9) did not produce any *spa* amplicon. The isolates were divisible into 7 *spa* types depending on the size as described in table 1. The amplicons obtained were 160 bp, 180 bp, 200 bp, 260 bp, 280 bp and 300 bp with calculated number of repeats of 5, 6, 6, 9, 10 and 11, respectively. All isolates produced a single amplicon except one (C8) which produced 2 amplicons. The amplicon of 300 bp was detected in 7 isolates followed by 260 bp amplicon in 6, 280 bp amplicon in 5 and 200 bp amplicon in 4 and remaining amplicons of 160 bp and 180 bp in 1 isolate each (Fig 1). The *spa* types obtained in the present study are similar to those reported by Rathore *et al* (2012) who observed amplicons of similar sizes in *S. aureus* isolates obtained from camel skin wounds and abscesses. Eventhough, they recorded presence of an additional 500 bp size amplicon but did not record any isolate without *spa* gene.

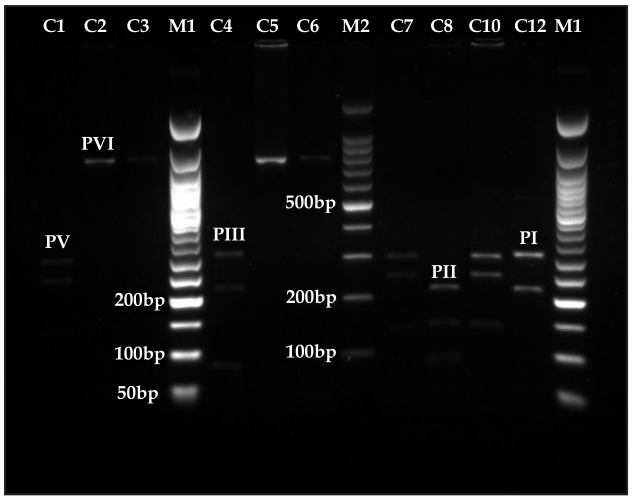

Fig 3. Different RFLP patterns of coa gene of *S. aureus* isolates C1 to C12, PI to PVI–Various RFLP patterns M1–50 bp DNA ladder, M2–100 bp DNA ladder.

The results in the present study are in conformity to those of Yadav *et al* (2015a) from the same area of study who reported that all their 32 *S. aureus* isolates obtained from milk of cattle (16) and buffalo (16) were divisible into 7 *spa* types with amplicon sizes ranging between 120 bp and 380 bp. The detection of 2 (260 and 300 bp) *spa* amplicons in one isolate in our study is in conformity to the observations of Shakeri *et al* (2010) and Bhati *et al* (2016). In the present investigation, one isolate did not produce *spa* gene amplicon which corroborated earlier observations of Kalorey *et al* (2007), Baum *et al* (2009), Momtaz *et al* (2010), Salem–Bekhit *et al* (2010), Khichar *et al* (2014) and Bhati *et al* (2016) who recorded non-*spa* typable strain of *S. aureus* and suggested that in these strains either *spa* mutation occurred or *spa* was absent. In the present study, 19 isolates were considered to be pathogenic since they possessed more than 7 repeats, as suggested by Frenay *et al* (1996). On the other hand, no correlation was reported between number of tandem repeats and pathogenicity of the isolates by other workers (Nashev *et al,* 2004; Kuzma *et al,* 2005; Jakubczak *et al,* 2007 and Kurlenda *et al,* 2010).

Variations in coa gene

All the 26 *S. aureus* isolates showed amplification of *coa* gene and they also produced free coagulase as detected in tube test. In our study, we obtained 5

coagulase types based on variable amplicon size where amplicons of 510 bp, 600 bp, 680 bp, 710 bp and 760 bp were produced by 4, 2, 4, 6 and 10 isolates, respectively (Fig 2; Table 2). The amplicon sizes obtained in the present study were much similar to that obtained by Salasia *et al* (2004) who carried out *coa* gene typing using the similar primers as in the present study, with *S. aureus* isolates from bovine sub-clinical mastitis. From the present study area Rathore *et al* (2012) reported 600 bp, 710 bp, 760 bp and 850 bp isolates from camels skin isolates, Khichar *et al* (2014) reported 510 bp, 600 bp, 710 bp and 850 bp amplicons from cattle mastitis isolates and Bhati *et al* (2014) reported 400 bp, 490 bp, 510 bp, 550 bp, 600 bp, 710 bp, 760 bp, 810 bp and 850 bp amplicons from cattle subclinical mastitis.

The above results indicated that of the 5 coagulase types in the present study three (600 bp, 710 bp and 760 bp) were similar to those reported by Rathore *et al* (2012) in camel wounds and abscesses isolates. The studies from the same area revealed that amplicon of 600 bp was produced by all the *S. aureus* isolates irrespective of type of infection and animal species from where the isolates were recovered. The amplicons obtained in the present study were also reported by one or more workers from the same area. A huge polymorphism in *coa* gene of *S. aureus* isolates from the area may be due to deletion or insertion mutations changing the *coa* gene size as suggested by El-Jakee *et al* (2010).

RFLP of coa gene

Restriction fragment length polymorphism has been found to be very useful tool in differentiating various strains based on digestion of amplicons with endonucleases resulting into fragments of variable lengths. Hookey *et al* (1998) were first to describe a coagulase based PCR RFLP technique that differentiated the major epidemic methicillin resistant *S. aureus* strains and minor epidemic strains. In the present investigation of the 5 coagulase types only 4 types (510 bp, 600 bp, 680 bp and 710 bp) were digested whereas 1 with 760 bp amplicon (from 10 isolates) remained undigested (Fig 3). The number of fragments produced upon *Alu*I digestion varied from 2 to 3 and their sizes varied from 80 bp to 300 bp. Overall six RFLP patterns (PI to PVI) were generated after digestion (Table 3). In our study, fragments of similar sizes were detected among different sized coagulase types *viz.* fragment of 80 bp was obtained with digests of 510 bp and 600 bp coagulase types, fragment of 150 bp was obtained with digests of 510 bp and 710 bp coagulase types, fragment of 210 bp was obtained with digests of 510 bp, 600 bp and 680 bp coagulase types, fragment of 260 bp size was obtained with digests of 680 bp and 710 bp coagulase types and fragment of 300 bp was obtained with digests of 510 bp, 600 bp and 710 bp coagulase types. The presence of similar fragments in different amplicons reveals common sites for endonucleases to act upon. On RFLP of *coa* gene similar fragments were also reported by some workers in mastitis isolates from the same area of study *viz.* Sanjiv *et al* (2008), Upadhyay *et al* (2012) and Bhati *et al* (2014) who reported fragments of 210 bp, 260 bp and 300 bp, Khichar *et al* (2014) reported fragments of 150 bp, 210 bp, 260 bp and 300 bp and Yadav *et al* (2015b) reported fragments of 80 bp, 150 bp, 210 bp and 300

Table 2. *Coa* gene variations in *S. aureus* isolates from camel skin wounds.

S. No.	Coagulase types	Isolates Numbers	Total isolates (%)	*coa* gene amplicon size (bp)
1.	1	C8, C12, C13, C22	4 (15.38)	510
2.	2	C4, C19	2 (7.69)	600
3.	3	C14, C15, C25, C26	4 (15.38)	680
4.	4	C1, C7, C9, C10, C16, C24	6 (23.07)	710
5.	5	C2, C3, C5, C6, C11, C17, C18, C20, C21, C23	10 (38.46)	760

Table 3. RFLP patterns of *coa* gene of *S. aureus* from camel skin wounds.

S.No.	RFLP Pattern	Isolates	Total isolates (%)	*coa* gene amplicon (bp)	RFLP fragment size (bp)
1	P I	C12, C13	2 (3.84%)	510	300, 210
2	P II	C8, C22	2 (3.84%)	510	210, 150, 80
3	P III	C4, C19	2 (3.84%)	600	300, 210, 80
4	P IV	C14, C15, C25, C26	4 (15.38)	680	260, 210
5	P V	C1, C7, C9, C10, C16, C24	6 (23.07)	710	300, 260, 150
6	PVI	C2, C3, C5, C6, C11, C17, C18, C20, C21, C23	10 (38.46)	760	760

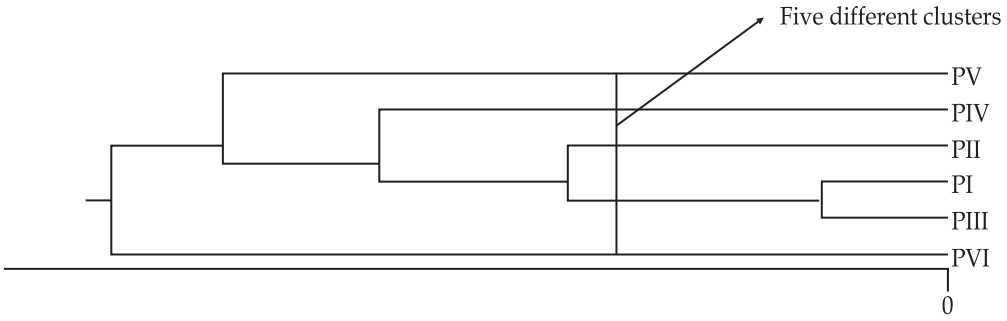

Fig 4. Cluster analysis of RFLP patterns of coa gene by dice and UPGMA method PI to PVI–different RFLP patterns.

bp. A variable RFLP patterns have been reported for *coa* amplicons with *S. aureus* isolates obtained from various origins. Schlegelova *et al* (2003) studied 86 *S. aureus* isolates from dairy cows and humans on a farm and from 10 different *coa* gene amplicon ranging between 650 bp to 1050 bp obtained 10 different RFLP pattern after *Alu*I digestion and their sizes varied from 80 bp to 490 bp.

In the present study *coa* amplicon of 760 bp was not digested with *Alu*I. This observation is in agreement with the findings of Lange *et al* (1999) who also did not observe digestion of three PCR products (580 bp, 650 bp and 1060 bp) from *S. aureus* of bovine mastitis origin. Similarly, da Silva and da Silva (2005) also observed non-digestion of two of the amplicons (579 bp and 602 bp) in isolates of cow mastitis origin. Yadav *et al* (2015b) also recorded non-digestion of one amplicon of 400 bp of mastitis isolates from cattle and buffalo.

Cluster Analysis of RFLP patterns

In the present investigation genetic relatedness among 6 RFLP patterns was calculated by Dice and UPGMA analysis method. Out of these 6 RFLP patterns 5 patterns were closely related of which pattern PI and PIII were closest. The maximum occurring (10 isolates) RFLP pattern PVI formed a separate cluster in the dendrogram (Fig 4).

In conclusion, the present study on 26 isolates of *S. aureus* from camel skin wound revealed that a great genetic difference existed among isolates in regard to both *spa* and *coa* genes. The genetic variability in *S. aureus* strains may be one of the reasons to exhibit difference in pathogenicity and refractory response towards treatment of *S. aureus* infections.

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