

MOLECULAR CHARACTERIZATION OF COAGULASE GENES OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM MASTITIC RIVER BUFFALOES

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ABSTRACT

Mastitis is a major dairy herd problem mainly caused by *Staphylococcus aureus*. The *Staphylococcus aureus* isolated strains from mastitic milk samples were subjected to molecular characterization of *coa* gene and phylogenetic tree (using MEGA6.1 software package) was constructed which revealed that the *coa* gene of *S. aureus* strains, could be grouped in two clades which were closely related to *S. aureus* isolates from Japan, India and Taiwan, while *S. aureus* isolates from Germany, UK and USA were distantly related. These results indicated the genetic relatedness of Pakistani isolates with other reported isolates from different parts of the world. All the Pakistani strains of coagulase gene were 99.98-100% similar while their divergence was 0.02%. The Pakistani strains were 99.9% similar and 0.07% divergent from Indian, Japanese, Taiwan, UK and German reported strains. These findings will also be helpful in future for designing suitable mastitis control strategies in the country.

Keywords: Buffalo, Mastitis, *Staphylococcus aureus*, Coagulase gene.

INTRODUCTION

Mastitis is an inflammation of the parenchyma of the udder which results in physical, chemical and pathological changes in it. Inflammation of udder may be caused by any kind of injuries such as physical trauma, chemical irritants, toxins or infectious agents. The bovine mastitis results in reduction of milk production (Hussain *et al.* 2012).

S. aureus is considered the most common causative agent of mastitis in dairy animals and its genetic variability has contributed to the emergence of distinct epidemiologic profiles of strains (Karimuribo *et al.* 2005). *Staphylococcus aureus* is responsible for variety of infections in human and animals (Bartlett and Hulten, 2010; Gu *et al.* 2013) and treatments become more difficult due to its emerging strains.

Pathogenesis of mastitis may be caused by extracellular toxins, enzymes and surface antigens (O'Riordan and Lee, 2004). Coagulase gene of *S. aureus* is considered an important virulence factor. Amplification of *S. aureus* coagulase gene (*coa*) has been recommended as an accurate method for identification of virulent strains of *S. aureus* (Morandi *et al.* 2010; Goh *et al.* 1992). Sequencing of the coagulase gene shows great diversity in *S. aureus* population (Costa *et al.* 2012). Information regarding the genetic diversity of *Staphylococcus aureus* isolated from mastitis in cow is available but such information regarding *S. aureus* from buffalo mastitis is limited (Firyal *et al.* 2009). Various studies described that bovine mastitis is caused by a wide

variety of *Staphylococcus aureus* genotypes (Smith *et al.* 2005) and *Staphylococcus aureus* from mastitis represents a genetic heterogeneity (Fournier *et al.* 2008).

The similar types of studies have been conducted in 1992, 1994 and 1998 however there was not a single report from Pakistan in buffaloes. Pakistan being the 2nd largest buffalo producing country in the world and having mastitis as a major disease, therefore the major mastitis causing bacteria *S. aureus* was targeted to see its genetic architecture based on an important gene Coagulase using Sanger's sequencing technique (that has not been followed in the previous studies). Then we compared our isolate with other reported isolates from all over the world to have better idea of its origin, characteristics and possible control measures. This is worth mention that we reported *S. aureus* coagulase gene sequences from Pakistani buffaloes first time. Therefore this study is very important from perspectives of Pakistan.

MATERIALS AND METHODS

Collection of milk samples: The present study was carried out in District Lahore (Punjab) and Bhimber (Azad Jammu and Kashmir), Pakistan. Lactating buffaloes disregards of the parity were selected while buffaloes in dry period and heifer were excluded. A total of 4144 quarters from 1036 buffaloes were screened for the presence of clinical and subclinical mastitis. Fifty dairy herds were screened (25 from each district). The data was collected regularly and categorized into three

groups (small, medium and large having 5 to 10, 11 to 30, and more than 31 dairy buffaloes, respectively) during the study period. Clinical mastitis was diagnosed on the basis of signs and symptoms while subclinical mastitis was diagnosed by California Mastitis Test (CMT) as discussed by Iqbal *et al.* (2004). All milk samples positive for clinical or subclinical mastitis were collected in sterilized screw capped vials and transported to the Microbiology Laboratory, Department of Epidemiology and Public Health, University of Veterinary and Animal Sciences, Lahore in a cooler to maintain temperature below 4°C. The samples were processed within 12 hrs after collection from the field.

Isolation and identification of *S. aureus*: Each of the collected samples was streaked on Staph-110 and sheep blood agar plates. Gram's staining followed by biochemical test, e.g., Catalase test, Coagulase test, Staphylect plus test and Mannitol fermentation were performed for the characterization of *S. aureus* isolates. Pure cultures of *S. aureus* were preserved in 20% glycerol and kept at -80°C till further use.

DNA Extraction and Quantification: DNA extraction was performed as prescribed by Genomic DNA Purification Kit (Thermo Scientific USA). DNA quantification was done with Nanodrop Thermo (Fermentas USA) and working aliquots of all extracted DNA samples were adjusted at same concentration level i.e. 50 ng/μL. Stock DNA samples were stored at -20 °C freezer.

Selection of Primers: Coagulase (*Coa*) gene was amplified using primers Coag-F, 5'AACAAAGCGGCCCATCATTAAG3' and Coag-R, 5'TAAGAAATATGCTCCGATTGTC-G3'(Kumar *et al.* 2011). Primer for gene Coagulase were synthesized from GeneWorks Australia.

Primers Optimization and PCR Amplification: DNA amplification was carried in thermocycler (Kyratc SC 200) with following cyclic conditions: Initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec for Coagulase gene and extension at 72°C for 30 sec. A final extension at 72°C was carried out for 5 min. All the PCR products were run on 1.2 % agarose gel stained with ethidium bromide and visualized under UV light in Gel Documentation System (BioRad USA).

Sequencing and Phylogenetic Analysis: Positive PCR products were precipitated with 70 % ethanol (80μL) and finally dissolved in 20 μL of double distilled deionized water. The representative strains of *coa* gene (11) were sent to Australian Genome Research Facility Ltd (AGRF), University of Adelaide for Sanger's sequencing. Sequences were edited using Codon Code Aligner and highest similarities of *coa* gene with that of other strains

were searched using the BLAST tool of GenBank, NCBI. (Woo *et al.* 2001). Phylogenetic trees of all sequences were constructed using MEGA6.1 by Neighbor Joining method (Sudhir and Sudhindra, 2000) with 1000 bootstrap values (Tamura *et al.* 2011). Alignments, pairwise distance estimations, and similarities/variations at nucleotides levels of all Pakistani sequences and those reported from NCBI GeneBank were also performed by using MEGA6.1 software.

RESULTS

Identification of *S. aureus* isolates from mastitic milk samples: A total of 4144 quarters from 1036 buffaloes were screened and 673 (106 clinical and 567 subclinical) milk samples from 508 buffaloes were examined for mastitis. Out of 106 clinical mastitis milk samples examined, 71 belonged to Lahore and 35 to Bhimber; whilst, out of 567 subclinical mastitis milk samples those were examined, 365 belonged to Lahore and 202 to Bhimber. Among these 673 milk samples, 437 isolates of Staphylococci were recovered. All these 437 isolates of Staphylococci were Gram positive, catalase positive and showed hemolytic property on 5% sheep blood agar. Out of these isolates, only 236 were confirmed coagulase positive *S. aureus*.

Amplification of PCR Products: The results of amplification of *coa* gene has been shown in figure 1. The PCR products were categorized into five classes on the basis of their molecular size on electrophoresis of agarose gel. The approximately the product size was from 430 to 840 bp. But the majority of isolates were having product size 575 bp.

Phylogenetic tree of *coa* gene of *S. aureus* isolates/strains from Pakistan and different geographical locations: Coagulase gene from *S. aureus* strains was amplified and PCR products of 430 to 840 bp were confirmed on agarose gel. PCR products were sequenced and nucleotide sequences were compared with other coagulase gene sequences in the GenBank. Phylogenetic tree was constructed by aligning coagulase gene sequences of present study isolates with selected coagulase gene sequences in the GenBank. Out of 236 samples we selected 11 samples for sequencing representing all major locations/ sampling pockets. There presentative coagulase gene of *S. aureus* strains were sequenced and phylogenetic tree was constructed. Phylogenetic tree of all Pakistani (n=11) sequences was constructed combined with all others sequences (n=9) in the NCBI GenBank database. Phylogenetic tree showed that all Pakistani isolates were grouped together in two clades with high bootstrap values showing high genetic relatedness with some isolates from Japan (GenBank accession numbers AB373752 and AB373753), while some isolates from India (JX240355) and Taiwan

(CP003603 and CP003166) were relatively distantly placed as compared to above mentioned Japanize isolates. Isolates from Germany (AJ306908), UK (FN433596 and BX571856) and USA (CP006044) were placed distantly as compared to isolates from Japan, India and Taiwan (Figure2).

Pairwise similarities and divergence among substitutions of Coagulase gene: The similarities and divergence among Pakistani and NCBI reported strains

from the world has been shown in table 1. All the Pakistani strains of coagulase gene were 99.98-100% similar while their divergence was 0.02%. The Japanizes strains were close to Pakistan strains and their similarity and divergence was 99.97% and 0.03%, respectively. The Pakistani strains were 99.93% similar and 0.07% divergent from Indian and German strains. Similarly, the Pakistani strains were 99.94% similar and 0.06% were divergent from UK and Taiwan reported strains.



Figure 1. Agarose gel electrophoresis of *coa* gene PCR amplification products. 100 bp DNA ladder, lane 1-2: 540 bp, lane 3,6: 550 bp, lane 4-5,10: 525 bp, lane 7: 430 bp and lane 9: 840.

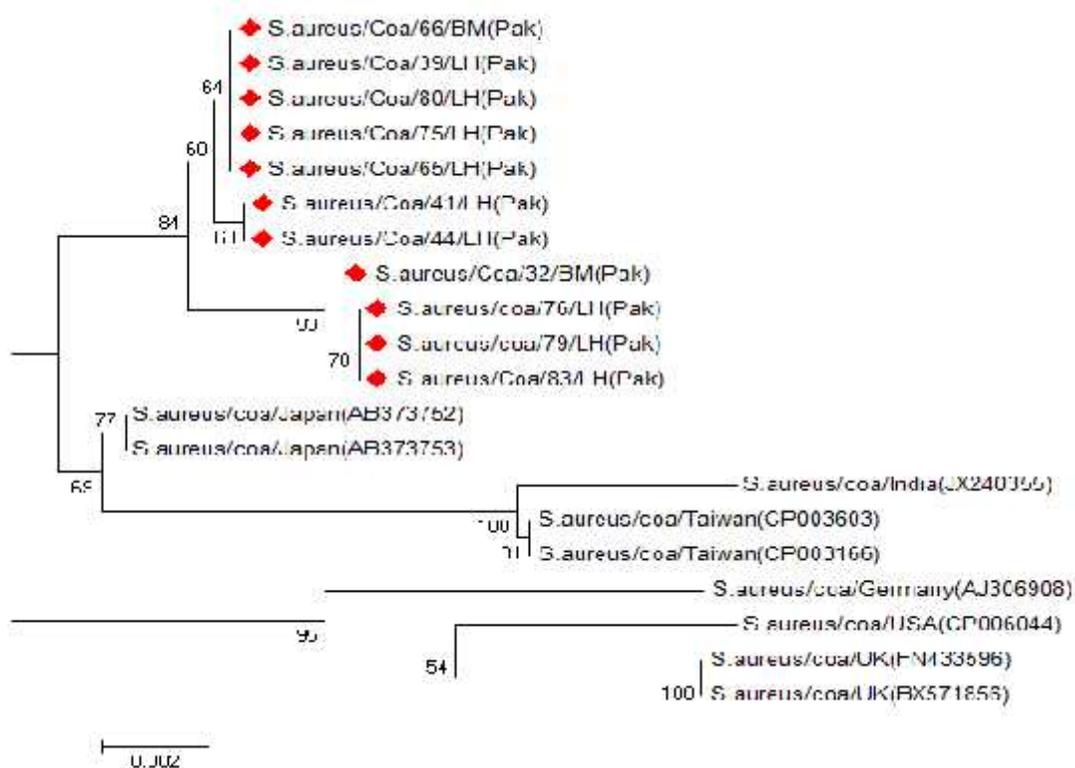


Figure 3. Phylogenetic tree (MEGA6.1, NJ method with 1000 bootstrap value), based on coagulase gene sequences, showing evolutionary relationship of Pakistani strains/isolates with selected reference strains from different geographical locations. Pakistani strains/isolates have been indicated with closed triangles.

Table 1. Pairwise genetic distance relationship between isolated strains and some reference strains based on coagulase gene sequence.

S. No.	Name of isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	S.aureus/coa/76/LH(Pak)		0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02
2	S.aureus/coa/79/LH(Pak)	0.00		0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02
3	S.aureus/coa/83/LH(Pak)	0.00	0.00		0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02
4	S.aureus/Coa/66/BM(Pak)	0.00	0.00	0.00		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02
5	S.aureus/coa/65/LH(Pak)	0.02	0.02	0.02	0.01		0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.02	0.02	0.01	0.01	0.02	0.02
6	S.aureus/coa/75/LH(Pak)	0.02	0.02	0.02	0.01	0.00		0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.02	0.02	0.01	0.01	0.02	0.02
7	S.aureus/coa/80/LH(Pak)	0.02	0.02	0.02	0.01	0.00	0.00		0.00	0.00	0.00	0.00	0.01	0.01	0.02	0.02	0.01	0.01	0.02	0.02
8	S.aureus/coa/32/BM(Pak)	0.02	0.02	0.02	0.01	0.00	0.00	0.00		0.00	0.00	0.00	0.01	0.01	0.02	0.02	0.01	0.01	0.02	0.02
9	S.aureus/coa/41/LH(Pak)	0.01	0.01	0.01	0.02	0.00	0.00	0.00	0.00		0.00	0.00	0.01	0.01	0.02	0.02	0.01	0.01	0.02	0.02
10	S.aureus/coa/44/LH(Pak)	0.01	0.01	0.01	0.02	0.00	0.00	0.00	0.00	0.00		0.00	0.01	0.01	0.02	0.02	0.01	0.01	0.02	0.02
11	S.aureus/coa/39/LH(Pak)	0.02	0.02	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00		0.01	0.01	0.02	0.02	0.01	0.01	0.02	0.02
12	S.aureus/coa/Japan(AB373752)	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02		0.00	0.01	0.02	0.01	0.01	0.01	0.02
13	S.aureus/coa/Japan(AB373753)	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00		0.01	0.02	0.01	0.01	0.01	0.02
14	S.aureus/coa/India(JX240355)	0.07	0.07	0.07	0.07	0.06	0.06	0.06	0.06	0.05	0.05	0.06	0.05	0.05		0.03	0.01	0.01	0.02	0.03
15	S.aureus/coa/UK(FN433596)	0.06	0.06	0.06	0.07	0.06	0.06	0.06	0.06	0.05	0.05	0.06	0.07	0.07	0.11		0.03	0.03	0.02	0.00
16	S.aureus/coa/Taiwan(CP003603)	0.06	0.06	0.06	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.03	0.03	0.02	0.10		0.00	0.02	0.03
17	S.aureus/coa/Taiwan(CP003166)	0.06	0.06	0.06	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.03	0.03	0.02	0.10	0.00		0.02	0.03
18	S.aureus/coa/Germany(AJ306908)	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.05	0.05	0.09	0.07	0.08	0.08		0.02
19	S.aureus/coa/UK(BX571856)	0.06	0.06	0.06	0.07	0.06	0.06	0.06	0.06	0.05	0.05	0.06	0.07	0.07	0.11	0.00	0.10	0.10	0.07	

The number of base substitutions per site from averaging over all sequence pairs within each group is shown. The diagonal bold faced numbers show the mean inter populational evolutionary diversity estimates.

DISCUSSION

Mastitis is common in dairy herds around the globe. It leads to economic losses and is a serious threat to animal and public health. Udder infection with *Staphylococcus aureus* considered to be the leading cause. Mastitis can be minimized by investigation of particular pathogens and their virulent factors involved in pathogenesis. The molecular characterization of pathogens is essential part to track the spread of contagious infections from one region to others or among dairy herds (Khan *et al.* 2013). With the development of molecular techniques and their application, now it is possible to differentiate between pathogenic and nonpathogenic strains of *S. aureus*. *Staphylococcus* is a normal inhabitant of skin and nares. The isolation of *Staphylococcus aureus* from milk alone is unequivocal to determine its role in the pathogenesis. *S. aureus* virulent gene surveillance could be helpful in detecting genetic diversity among these major mastitis causing pathogens. Total 11 strains of coagulase gene of *S. aureus* isolated from mastitis cases were further characterized by PCR amplification of virulent genes. As the sequence similarity was very high among the samples therefore we selected a few representative samples from each country/ region.

In present study the identification of virulent strains coagulase were targeted. Coagulase enzyme activity is the principle criteria for diagnosis of *S. aureus* from other coagulase negative *Staphylococcus* because coagulase can convert fibrinogen to fibrin.

In *S. aureus* identification, coagulase production (coagulase test) has been considered as an important criterion for phenotypic identification (Upadhyay *et al.* 2012; Gharib *et al.* 2013). In molecular investigation coagulase gene amplification has been considered as a most accurate method for *Staphylococcus aureus* typing. The similar types of studies have been conducted in 1992, 1994 and 1998 however there was not a single report from Pakistan in buffaloes previously. Therefore the major mastitis causing bacteria *S. aureus* was targeted to see its genetic architecture based on an important gene coagulase. In case of molecular identification, the presence of coagulase gene is highly polymorphic and does not give similar amplicons in amplification. This amplicon difference in coagulase gene size is due to that 3' end contains a series of 81bp tandem repeats and number of which may differ between strains (Upadhyay *et al.* 2012; Goh *et al.* 1992). Coagulase gene consists of three distinct regions: (i) the N-terminus containing the prothrombin-binding site, (ii) a central region which is highly conserved, and (iii) a C-terminal region, each encoding 27-amino acid residues (Janwithayanuchit *et al.* 2006).

Present study showed that prevalence of coagulase positive *S. aureus* was quite common in both localities studied (i.e., Lahore and Bhimber) and

molecular characterization of *S. aureus coa* gene showed genetic variation. Similarly, prevalence of *S. aureus* mastitis in buffaloes is of great concern in Asian countries as these animals are major contributor to milk industry (El-Jakee *et al.* 2010). Present study is agreed with previous study that PCR amplifications of *coa* gene, revealing extensive polymorphism with predominance of one or more of *coa* gene amplified products responsible for mastitis in buffaloes (Sindhu *et al.* 2010). The phenotypic and genotypic variation was observed in some of the cases. These observations are supported by previous reports as mutation in the coding sequence or plasmid number (Kumar *et al.* 2010). Virulence factors diversity and their various combinations might cause change in the level of pathogenicity, and spreading of infections within and between animals (Kumar *et al.* 2010). The end region of *coa* gene differs among *S. aureus* isolates, both in their number and in location (Goh *et al.* 1992), so the coagulase is an important feature used worldwide for epidemiological investigations of *S. aureus*. It is also the easiest gene to analyze polymorphism and generates multiple distinct polymorphism patterns.

The present study results showed heterogeneity in the *coa* gene of *S. aureus* strains in Pakistan and it was in accordance with the heterogeneity reported in other strains of *S. aureus* (Khan *et al.* 2013; Momtaz *et al.* 2011). Less variation in *coa* gene of *S. aureus* was found in the present study, which agrees with Mork *et al.* (2005) that small number of closely related genotypes of *S. aureus* are responsible for mastitis in a locality. The coagulase gene is polymorphic and genetically variable and this polymorphism is due to multi allelic forms on the 3' end of the gene which differs in their sequence and restriction sites (Wisal *et al.* 2005). The PCR amplicons variation in size of coagulase gene could be due to polymorphism among different isolates obtained from different herds and previous studies have also confirmed PCR product variation using molecular analysis of the coagulase gene (Khan *et al.* 2013); however, reason of this polymorphism is still unknown (Saei *et al.* 2009). The present study agreed with the previous study conducted by Khan *et al.* (2013) in Pakistan that this polymorphism may be due to mutations by which different nucleotides are inserted or deleted particularly in 3' end the coagulase gene. The different *coa* gene products and their polymorphism for *Staphylococcus aureus* demonstrate great genotypic variability among the organisms (Sanjiv *et al.* (2008).

In the present study, coagulase strains produced five types of amplicon (430, 525, 540, 550 and 840 bp) and only one was dominant (525bp). These five types of amplicons are in accordance to findings of Sanjiv *et al.* (2008) reported that a single *coa* genotype was dominant from 5 genotypes and PCR amplification produced 400, 500, 600, 900 and 1000-bp amplicons with 600-bp being

the most common in a geographical region. In present study, PCR products size ranged from 430 to 840 bp which is close in size from PCR product variation (500 to 580 bp) reported by Hookey (1998), confirming the polymorphic nature of coagulase gene found in different strains of *S. aureus*. In 2004, similar finding was observed by Salasia *et al.* in which amplicons of 510, 600, 680, 740, or 850 bp size were recorded and only one was found dominant. Momtaz *et al.* (2011) suggested that mastitis in cattle is caused by certain strains of *S. aureus*, mostly carrying a coagulase gene, and this information might be helpful for control and management of mastitis caused by *S. aureus*.

Detection of major and minor strains of mastitis causing pathogens is always helpful. These information regarding molecular characterization of coa gene of *S. aureus* could be helpful in prevention and control of infection in dairy animals.

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