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Characterization of *Staphylococcus aureus* by pulsed field gel electrophoresis

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ABSTRACT

Staphylococcus aureus is reported as principal cause accounting for more than 19 to 40% of the cases of bovine mastitis. The pulsed field gel electrophoresis (PFGE) which is currently considered as the gold standard method was used in the present study for characterization of *S. aureus*. *S. aureus* isolates (29) recovered from bovine mastitis milk samples were subjected to the PFGE. Out of 29 isolates, 22 could be digested by *Sma*I enzyme and yielded dendrogram showing 2 major clusters with 8 pulsotypes (A to H) having > 60% similarity coefficient. The pulsotype A, C, D and F were constituted of isolates recovered from both cows and buffaloes, whereas pulsotype B and H were from buffaloes only and pulsotype E and G were from cattle origin. The pulsotype A isolates were coagulase negative while all the isolates of pulsotype F and H were coagulase positive; and pulsotype B, C, D, E and G were having both coagulase positive and negative isolates. No correlation between geographical area and other virulence factors was observed, since isolates belonged to diverse origin of locations from 2 different districts of western Maharashtra. Thus, PFGE proved to be a good tool for genetic discrimination of *S. aureus* strains in molecular epidemiology.

Key words: Bovine mastitis, Characterization, Pulsed field gel electrophoresis, *Staphylococcus aureus*

Bovine mastitis has been a major challenge to the worldwide dairy industry despite the widespread implied control strategies (Gruet *et al.* 2001, Bradley 2002, Viguier *et al.* 2009). Mastitis continues to be the most economically important disease of dairy cattle, accounting for 38% of the total direct costs of the common production diseases. Among the main organisms responsible for bovine mastitis, *Staphylococcus aureus* is the primary and probably the most disastrous agent, because it causes chronic infection of the mammary gland which then makes it difficult to carry out prophylactic measures (Miles *et al.* 1992) and it accounts for more than 19 to 40% of the cases of bovine mastitis (Kumar 2004).

Pulsed field gel electrophoresis (PFGE), currently considered the gold standard method, is highly reproducible, discriminatory and is being widely applied in typing of bovine *S. aureus* (Zadoks *et al.* 2002, Jorgensen *et al.* 2005). This technique results in the separation of DNA fragments up to ~10 Mb; helps in discriminate among community- and health care-acquired ORSA (oxacillin resistant *Staphylococcus aureus*) strains (Mcdoughal *et al.* 2003); and is also useful in studying the relationship between molecular techniques. Pulsed-field gel electrophoresis is a highly discriminatory and sensitive technique for distinguishing strains of *S. aureus* and has been used for micro epidemiologic (local or short-term) and macro epidemiological (national, continental, or long-term) surveys (Ciftci *et al.* 2009). Hence, pulsed field gel electrophoresis (PFGE) was used in the present study for characterization of *S. aureus*.

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MATERIALS AND METHODS

Pulsed field gel electrophoresis of S. aureus: *Staphylococcus aureus* isolates (29) recovered from bovine mastitis milk samples were subjected to pulsed field gel electrophoresis. Genomic DNA fingerprints of the *S. aureus* isolates were determined using pulsed-field gel electrophoresis (PFGE) as per the standard protocol developed by Pulse-Net website for *S. aureus*

Preparation of blocks: A single colony of *S. aureus* was inoculated in 10 ml of BHI broth and incubated in shaker incubator at 160 rpm speed at 37°C for 24 h. The culture was diluted 1: 10 proportion in BHI broth and centrifuged at 5,000 rpm for 10 min. The supernatant was discarded and cell were resuspended in 1ml TE buffer and then centrifuged for 4 min at 15,000 rpm. Supernatant was discarded. This step was repeated twice and then the cells re-suspended in 500 µl of EC lysis buffer. Molten agarose (1.8%) was added in 500 µl quantity into the cells and 100 µl of cell-agarose mix was pipetted to labeled blocks. Blocks were allowed to solidify and each sample consisted of 2 blocks. Blocks were placed in lysine buffer (lysis buffer 836 µl, lysostaphin 64 µl, and lysozyme 100 µl) and

Table 1. Isolates showing PFGE clusters with their origin

Cluster No.	Pulso type No.	Isolates from cattle origin	Isolates from buffalo origin
I	A	01 (coa -ve)	01 (coa-ve)
	B	00	01 (coa+ve)
II	C	00	08 (coa+ve and -ve)
	D	02 (coa+ve and -ve)	02 (coa+ve and -ve)
	E	01(coa –ve)	00
	F	00	02 (coa+ve)
	G	01(coa+ve)	00
	H	00	03 (coa+ve)

incubated at 37°C for 2 h in shaker incubator @ 160 rpm/ min. Blocks were washed twice in TE buffer and 4 times by distilled water. After discarding lysis buffer, 2–4 ml of de - proteinising buffer was added and incubated at room temperature for 1 h. The entire procedure was repeated again. After the second incubation de-proteinising buffer was pipetted out, 800µl - de-proteinising buffer + 100µl – proteinase K + 100µl – Sarcosyl (0.1mg/ml) was added and incubated at 55°C in water bath for 2–3 h. De-proteinising buffer pipetted out completely, 3 ml TE buffer was added and incubated at room temperature for 3 h. The buffer was pipetted out; 3ml of fresh TE buffer was added and stored at 2–8°C for use.

Restriction enzyme digestion reaction: Each of the 29 tubes with *S. aureus* isolate plugs was labeled and restriction buffer was added to them. One block of each isolate was taken, placed in the tube containing restriction buffer and incubated at room temperature for 30 min. The restriction buffer was pipetted out and then fresh 150 ml restriction buffer (Tango buffer) was added with 4µl (10U/µl) restriction enzyme *Sma*I. The tubes were then incubated at 25°C in water bath for 4 h and enzyme restricted *S. aureus* isolates' plugs were loaded in 1% agarose prepared in TBE buffer.

Running of PFGE gel: Lambda marker used as a standard was applied in the last well of the gel by taking care of avoiding air bubble between the block and the gel. The empty spaces between well and fitted block were filled with 1.8% low melting gel to avoid smearing.Freshly prepared buffer was used, gel was run at 6V voltage with initial 5 and final 50 sec pulse time and further for 21 h. After 21 h run, gel was stained with ethidium bromide and examined under UV light.

The PFGE patterns generated were analysed and dendrogrammed by using the Gel Compare II software. The pattern of clustering was produced using unweighted-pair group algorithm and the Dice correlation coefficient with a tolerance of 1%. Results of clustering analysis were confirmed by comparing with the PFGE profiles. A similarity coefficient of 60% was selected to define the pulsed-field type clusters.

RESULTS AND DISCUSSION

The molecular sub-typing methods allow the identification and tracking of pathogen accurately. The pulsed field gel electrophoresis (PFGE) which is currently considered as the gold standard method is highly reproducible, discriminatory and is widely applied in typing of bovine *S. aureus* (Zadoks *et al.* 2002, Jorgensen *et al.* 2005). PFGE is a molecular fingerprinting technique used to classify bacteria based on restriction sites within the bacterial genome beyond the species level.

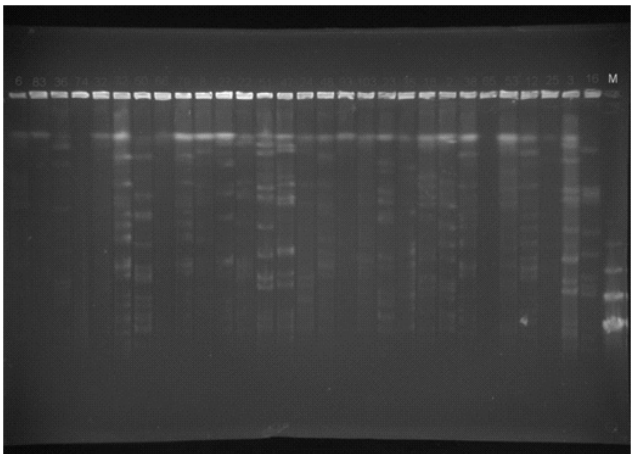

Fig. 1. PFGE pattern of *S. aureus* isolates digested by *Sma*I restriction enzyme. 1: B6, 2: B83, 3: C36, 4: B74, 5: B32, 6: C72, 7: B60, 8: C66, 9: B79, 10: B8, 11: B27, 12: B22, 13: B51, 14: B47, 15: B24, 16: C48, 17: C93, 18: B103, 19: B23, 20: C35, 21: B18, 22: B2, 23: B38, 24: B65, 25: C53, 26: B12, 27: B25, 28: B3, 29: C16, 30: PFGE Marker I (Lambda marker I).

Most of the isolates recovered were coagulase, hemolysin and DNAse producing strains along with multidrug resistance property. These isolates with different virulence factors and originated from different locations (Kolhapur and Sangli district) of Maharashtra were subjected to pulsed field gel electrophoresis. Out of 29 isolates subjected to PFGE, 22 could be digested by *Sma*I enzyme and produced good restriction and band pattern (Fig. 1). Therefore, 22 enzyme digested isolates showed good imaging with different banding patterns under the gel illuminator, thereby yielding dendrogram of only those isolates (Fig.2). The dendrogram of remaining 7 isolates could not be obtained due to non-digestion of DNA of these isolates. Similar results of inability of *Sma*I enzyme digestion of DNA were reported by Bosch *et al.* (2010).

Based on 60% similarity coefficient, 2 clusters (I and II) and 8 pulsotypes (A to H) were produced. Cluster I consisted of 2 pulsotypes (A and B) with 65% similarity coefficient. Amongst the 6 pulsotypes of cluster II, pulsotype C and pulsotype D were having 78% similarity coefficient and these 2 pulsotypes showed 75% similarity with pulsotype E. Pulsotypes F, G and H exhibited 75% similarity coefficient. Of these, pulsotype F and pulsotype G were related with 80% similarity coefficient.

The pulsotype A, C, D and F were constituted of isolates from both cows and buffaloes, whereas pulsotype B and H were produced from buffalo's isolates only and pulsotype

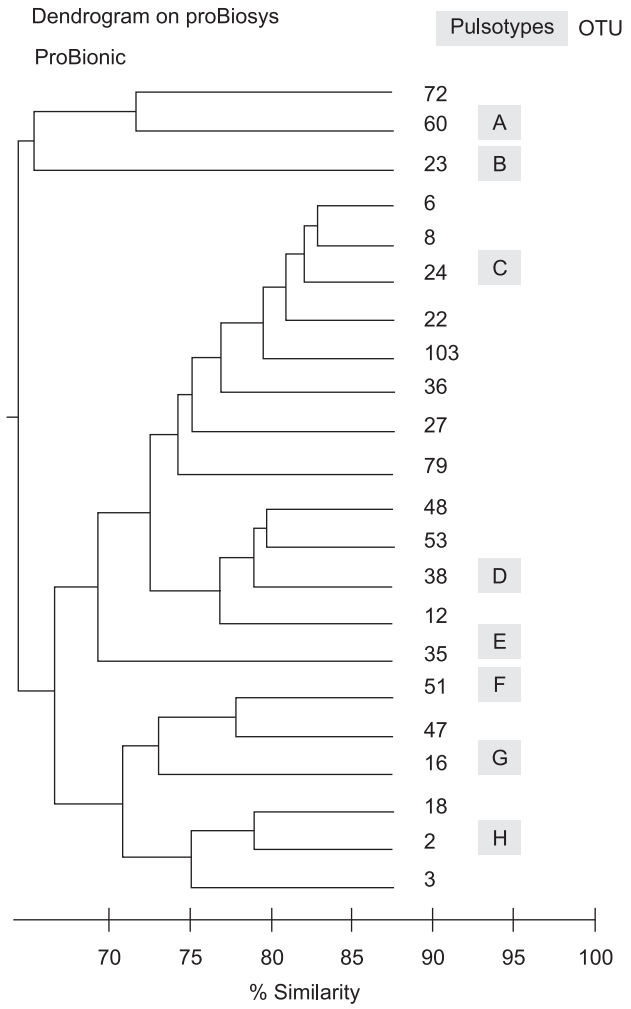

Fig. 2. Dendrogram of PFGE image of *S. aureus* isolates.

E and G were of isolates from cattle origin. The isolates of pulsotype A were coagulase negative while all the isolates of pulsotype F and H were coagulase positive; whereas, pulsotype B, C, D, E and G were having both coagulase positive and negative isolates (Table 1).

Andrea *et al.* (2010) observed the relationship among coagulase (*coa*) gene amplification and their PFGE pattern. Moreover, similar findings of different cluster and pulsotype formation were observed by various authors based on different geographical regions. Akineden *et al.* (2001) observed 12 different PFGE patterns during the macrorestriction with *Sma*I. Zadoks *et al.* (2002) observed 24 main types and 7 subtypes from the samples of various farms. Mc Dougal *et al.* (2003) used *Sma*I-digested genomic DNA separated by PFGE to characterize 957 *S. aureus* isolates and established a database of PFGE patterns. A dendrogram of percent similarity, calculated with Dice coefficients from the PFGE data using a cutoff of 60–80 %, revealed 8 major clusters of isolates. Aires-de-Sousa *et al.* (2007) found 30 mastitis causing *S. aureus* isolates grouped into 5 clusters irrespective of phenotypic property. Ciftci *et al.* (2009) found 19 pulso-types from 47 *S. aureus* isolates from bovine mastitis cases; of which 12 pulsotype contain single isolates and also observed that all the pulsotypes were unique to the geographical locations. Oliveira *et al.* (2011) identified 12 pulsotypes and 5 subtypes among the 83 isolates; 5 of the 12 pulsotypes were represented by only single isolate. These authors reported differences in their PFGE pattern and thereby genetic variability amongst *S. aureus* isolates, due to differences in geographical origin of isolates. Our results correlated with the reports of above authors.

The dendrogram revealed that, cluster and pulsotype differentiation was based on to some extent coagulase production (Andrea *et al.* 2010). Analysis of the results of PFGE patterns of the field isolates did not reveal any correlation between pulsotypes and species affected or pulsotype and geographical location, since isolates were recovered from diverse origin of locations from 2 different districts. Akineden *et al.* (2001) and Bosch *et al.* (2010) reported similar findings on genetic variability and also reported the variability amongst isolates with the possibility of diverse origin of the samples collected under their genetic investigational pattern of isolates. Whereas, genetic relatedness or similarity in PFGE pattern of isolates collected from same farms was observed by Haveri *et al.* (2008) and Ciftci *et al.* (2009). Thus PFGE is found to be a good tool for genetic discrimination of *S. aureus* strains in molecular epidemiology.

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