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Application of pulsed field gel electrophoresis for molecular characterization of vancomycin resistant *Staphylococcus aureus* isolates from hospitals

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Abstract

Aim : The present study was conducted to characterize VRSA isolates on the basis of pulsed field gel electrophoresis (PFGE) and the presence of *spa* gene, recovered from different Doon Valley Hospitals.

Methodology : Six VRSA isolates were analyzed using PFGE and spa typing. *spa* gene coded Protein A was used as a genetic marker for the characterization of *Staphylococcus* aureus isolates. Dendrogram were constructed on the basis of unweighted pair group method with arithmetic means (UPGMA method) for clusters analyses.

Results : Dendrogram finally showed two major banding patterns at about 85% similarity designated as PFGE type A and PFGE type B exhibiting differences of 4-6 bands. The length of *spa* gene varied from 1200 to 1500 bp, showing variation in length. The most prevalent length was 1200bp.

Interpretation : VRSA clones are closely related as PFGE results showed less diversity. Only three strains exhibited *spa* gene.

Key words: Pulse field gel electrophoresis, Spa gene, *Staphylococcus aureus,* Vancomycin resistant, VRSA clones

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Introduction

Staphylococcus aureus is one of the most important pathogens causing nosocomial infections. In early 1960s, *S. aureus* adaptiveness to antibiotics led to the emergence of methicillin-resistant *S. aureus* (MRSA). The glycopeptide vancomycin was observed as prime option for treating multi drug resistant MRSA (Sievert *et al.,* 2008). However, there are several reports that indicate the appearance of vancomycin-resistant strains from different countries. However, the first vancomycin-resistant *S. aureus* (VRSA) strain was reported in Michigan in 2002 (Chang *et al.,* 2003). Later, various other cases of VRSA were reported in the United States (CDC, 2004; Weigel *et al.,* 2007), India (Tiwari and Sen, 2006; Saha *et al.,* 2008; Thati *et al.,* 2011) and Iran (Aligholi *et al.,* 2008; Dezfulian *et al.,* 2012). High level of resistance to vancomycin is due to the activity of van operons.

These operons produce D-Ala D-lactate (Lac), or D -Ala-D -serine is altered building block for peptidoglycan with much less affinity to glycopeptides and removal of normal D-Ala-D-Ala-ending precursors (Périchon and Courvalin, 2009). *S. aureus* pathogenesis has been assigned to produce a diverse range of proteins. *Staphylococcal* surface protein known as protein A 40-60 kD, protects the bacteria from host defense mechanism. *spa* gene encode Protein A composed of approximately 2,150 bp and harbours a number of functionally distinct regions which include, Fc-binding region of immunoglobulin G (IgG), X-region and C-terminus (Schneewind *et al.,* 1992).

The IgG Fc-binding region consists of five 160 bp repeats. The repetitive X region portion containing 24-nucleotide region is highly polymorphic and is related to the number and sequence of repeats. Variability of X region causes protein A difference (Shakeri *et al.,* 2010). Apart from its use as a marker, the number of repeats in the region X has been linked to the dispersal potential of MRSA, with greater numbers of repeats concerned with higher epidemic ability (Montesinos *et al.,* 2012). PFGE has become the most familiar tool for the prompt discrimination of MRSA strains due to its convenience and accuracy (Tenover *et al.,* 1997). PCR assay can improve its discrimination power in combination with PFGE (Walker *et al.,* 1998). The aim of this study was to investigate molecular characterization of VRSA isolates in hospitals using PFGE and molecular detection of *spa* gene, which enhances virulence potential.

Materials and Methods

Collection and isolation of VRSA strains: Specimens of patients of different wards were inoculated on mannitol salt agar (MSA) and incubated at 37°C for 48 hrs. Mannitol fermenting cultures were sub-cultured on nutrient agar to perform Gram's staining, catalase and coagulase test (Talwar *et al.,* 2016). The positive isolates were subjected to Oxacillin screen agar test for methicillin resistance. MIC of vancomycin for all methicillin resistant strains was determined by tube dilution method (macrobroth dilution). Two-fold dilutions of vancomycin ranging from 0.25-128 µg ml^{-1} were prepared in Mueller-Hinton broth (HiMedia, Mumbai). The inoculum was prepared by adjusting the concentration of strains to 10^5-10^6 colony forming units (CFU) ml^{-1} using McFarland standard and added to each tube of vancomycin and incubated for 24 hrs at 35°C. A high level of resistance (>32 µg ml^{-1}) against vancomycin was noted in 6 isolates (Talwar *et al.,* 2013). These isolates were vancomycin resistant. The demographic information of patients harbouring VRSA are represented in Table 1.

DNA purification and gel electrophoresis: Single colonies of the VRSA isolates were cultured in Brain Heart Infusion (BHI) broth and incubated for an overnight at 37°C. DNA purification was carried out. PFGE (pulsed field gel electrophoresis) was done as described by McDougal *et al.* (2003), with minor modifications as described in Arakere *et al.* (2005). The plugs were digested with SmaI restriction enzyme for 3 hr. Restriction fragments were separated on a 1•5% gel with an initial switch time of 5 sec and final switch time of 35 sec, voltage of 6 V/cm, included angle of 120° and running time of 21 hr using the CHEF-DRIII device (Bio-Rad). After electrophoresis, the gels were stained using ethidium bromide, destained in water and photographed under UV light with the Gel Doc system.

Spa gene detection

DNA extraction and Oligonucleotide primer sequence: PCR was performed for spa typing of various strains with 5'-ATCTGGTGGCGTAACACCTG-3' (forward) and 5'-CGCTGCACCTAACGCTAATG -3' (reverse) (Strommenger *et al.,* 2008).

PCR amplification: The PCR mixture consisted of 1 mmol l^{-1} magnesium chloride, 0.2 mmol l^{-1} dNTPs, PCR buffer, 1 µmol l^{-1} of primers, and 1 unit of Taq-DNA polymerase in a final volume of

Table 1: Demographic information of patients harbouring VRSA strains

Strain no	Sample	Ward	Gender	Age
MIMM22	Nasal	General	M	21
HHMM56	Wound	Burns	F	68
VNMM77	Nasal	General	F	21
CHMM114	Nasal	Dialysis	M	62
HHMM208	Nasal	Dialysis	F	64
DHMM267	Nasal	Burns	M	11

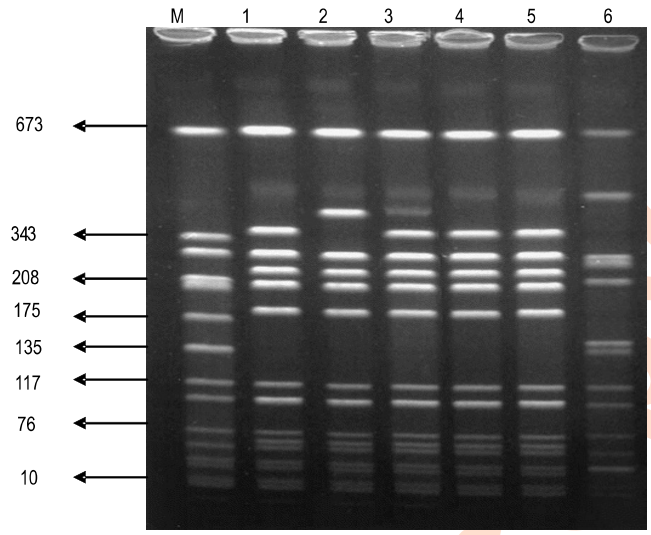

Fig.1: PFGE patterns of SmaI digests of MRSA isolates. Lanes M NCTC 8325; Lanes 1-6 indicate strains no.1-6. (band size in bp-base pair).

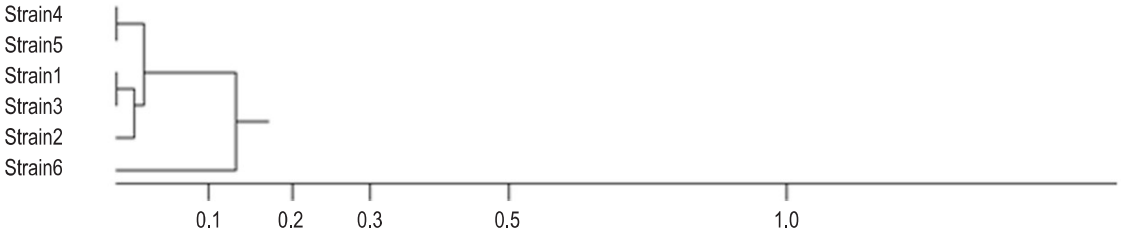

Fig. 2: Dendrogram based on the similarities derived from UPGMA.

50µl. Samples were denaturated at 94°C for 4 min followed by 35 cycles using the following parameters: denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 3 min, with a final extension at 72°C for 5 min.

Statistical analysis: Dendrogram were constructed on the basis of unweighted pair group method with arithmetic means (UPGMA method) for clusters analyses.

Results and Discussion

PFGE of smaI digested bacterial chromosomal DNA produced approximately 13-15 fragments ranging from 10-673 kb (Fig. 1). The dendrogram generated with standard clustering software (the unweighted pair group method with arithmetic means, UPGMA) (Fig. 2) showed two major banding patterns at about 85% similarity designated as PFGE type A and PFGE type B exhibiting differences of 4-6 bands. PFGE type A was further divided into three subtypes A1, A2 and A3. Subtypes A1 included strains 4 and 5, Subtype A2 represented strain 1 and 3 and Subtype A3 referred as strain 2. Strains 4 and 5, and strains 1 and 3 were most closely related with more than 96% similarity. PFGE type B include only strain 6 which showed different band pattern from the rest. In the present study, PFGE analysis showed majority of the isolates depicted dominant profile A. The subtypes A2 and A3 were found different, though with minor variations. Such minor genomic alteration can be explained by simple insertions or deletions of DNA or gain or loss of restriction sites (Tenover *et al.,* 1994). The *spa* gene detected in three out of six strains with no. 1, 2 and 3 varied between 1200 and 1500 bp in size. Only strain no. 3 had 1500 base pair band, while strain 1 and 2 had 1200 bp gene. Thus,

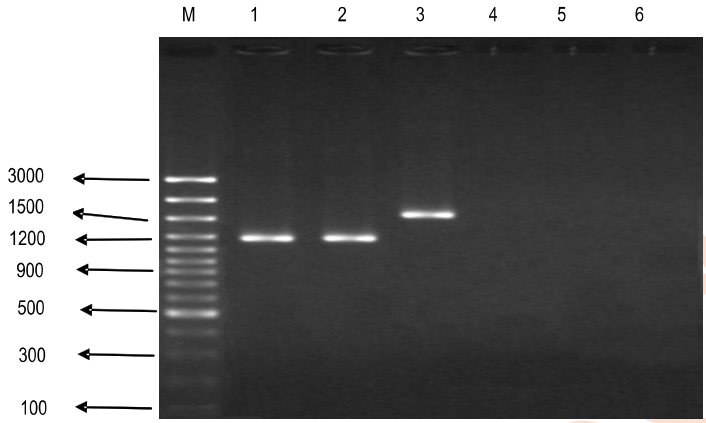

Fig. 3: Agarose gel electrophoresis of PCR-amplified product of spa gene. Lanes M (Molecular ladder), Lanes 1-6 indicate strains no.1-6 (band size in bp-base pair).

the band, length varies from 1200-1500 bp among all patients (Wichelhaus, *et al.* 2001). The remaining isolates did not show any band pattern, indicating lack of *spa* gene. In the present study, spa gene was expressed in only 3 isolates concerned (50%), and spa bands obtained were either equal to or greater than 1200 bp making strain capable of attaching firmly to epithelial cells which promote colonization and distant infection. *S. aureus* strains with shorter length of protein A cannot attach to the nasal epithelium surface and are released through breath, sneeze, and cough (Shakeri *et al.,* 2010). Strains with longer spa bands (1200 bp or more) are firmly adhered to the respiratory epithelium, which prevent their discharge through breath, sneeze and cough (Omar *et al.,* 2014). *spa* bands carrying strains are more frequently isolated from patients rather than from healthy carrier (Shakeri *et al.,* 2010).

Also, the *spa* gene carrying isolates were closely related in PFGE pattern. The pathogenicity of Protein A is due to attachment to Fc Domain IgG, complement fixation and so on. Spa gene is not regularly expressed in all *S. aureus* isolates (Frénay *et al.,* 1994). In few studies, the absence of spa gene in some *S. aureus* isolates have been reported. (Shakeri *et al.,* 2010 ; Adesida *et al.,* 2006). PFGE typing is useful to confirm MRSA outbreaks and strain relatedness (Tenovar *et al.,* 2009). Although, PFGE identifies minor variants among isolates as it accumulates variation rapidly, the main disadvantages are high initial cost due to the equipment required and the time-consuming procedure. The present findings indicate the existence of closely related VRSA clones widely spread in different Doon Valley hospitals. The isolates were grouped into two predominant PFGE types. The spa gene were found prevalent in half of the isolates with two different band sizes of 1200bp and 1500 bp. The present study provides a baseline for further characterization of *S. aureus* in the region.

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