American Journal of Infectious Diseases 5 (2): 163-169, 2009
ISSN 1553-6203


# Molecular Analysis of Coagulase Gene Polymorphism in Clinical Isolates of Methicilin Resistant *Staphylococcus aureus* by Restriction Fragment Length Polymorphism Based Genotyping

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**Abstract: Problem statement:** To investigate coagulase gene variants through Restriction Fragment Length Polymorphism (RFLP) amongst clinical isolates of Methicillin-Resistant *Staphylococcus Aureus* (MRSA) collected from Chennai based hospitals. **Approach:** A total of 85 MRSA clinical isolates collected from various clinical sources were studied for the molecular typing purpose. To perform coagulase gene typing, the repeated units encoding hypervariable regions of the *Staphylococcus aureus* coagulase gene were amplified by the PCR followed by AluI and HaeIII restriction enzyme digestion of the PCR product and analyzed for RFLP. **Results:** Cluster analysis performed with Un-weighted Pair Grouping Using Arithmetic Mean Analysis (UPGMA) at >80% similarity level classified all strains into 31 and 21 genotypes based upon AluI and HaeIII RFLP patterns respectively. AluI RFLP patterns gave a high discriminatory index and proved to be better than that of HaeIII in typing of MRSA strains. **Conclusion:** Method investigated in this study, proved to be quick, reliable and useful for typing large number of MRSA strains from various clinical isolates with high fidelity. Grouping of strains based on RFLP patterns of both enzymes, improved the discriminatory index. Finally, using this methodology group of epidemiological strains can be typed reliably and effectively using both enzymes. Significance and impact of the study: This efficient and reliable typing procedure is beneficial to develop efficient infection control measures in hospitals for staphylococcal infection. This typing procedure could be used to analyze large number of strains with in a short period of time and thus useful for epidemiological investigations.

**Key words:** Methicillin-resistant *staphylococcus aureus*, coagulase gene, molecular typing, Restriction Fragment Length Polymorphism (RFLP), Polymerase Chain Reaction (PCR)

## INTRODUCTION

*Staphylococccus aureus* is the major cause of nosocomial and community acquired infections. They are transmitted easily among patients and visitors. The drug resistant strains are arising rapidly and thus making the treatment difficult. As a result, Methicillin Resistant *Staphylococcus Aureus* (MRSA) is one of the leading cause of increasing morbidity and mortality. Rapid identification of infected patients and interruption of strain transmission is very crucial in controlling the spread of infection.

Rapid and accurate typing of *Staphylococcus aureus* is crucial to understand the transmission of this infectious organism. The traditional phenotypic methods have several drawbacks[1-3]. Bacteriophage typing for *Staphylococcus aureus* has been used internationally since 1951 and remains a cost- effective approach to typing the large number of referred isolates but it has some limitations[4]. The reagents for bacteriophage typing are not commercially available in certain parts of the world and hence MRSA strains are non-typeable with phages methodology. Other methods like plasmid analysis has several drawbacks, e.g., (1) Plasmids may be absent from isolates, (2) May vary in size, (3) May be readily lost during replication. Another method namely antibiogram schemes are often uninformative and many strains are acquired drug resistant[5]. Recently, several investigators have reported DNA-based techniques for typing strains and such molecular typing methods are based on stable genetic information[6-9]. Pulse Field Gel Electrophoresis (PFGE) and Multi Locus Sequence Typing (MLST) are proved to be efficient techniques in typing MRSA[10]. These typing method are technically complex, laborious, time consuming and expensive to be used in

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a clinical setting. It is well known that enzyme coagulase is produced by almost all strains of *S. aureus*. Its production criterion is now being used in the clinical microbiology laboratory for the identification of *S. aureus* in human infections. It is now proved that coagulase is an important virulence factor during infection process. The coagulase gene amplification has been considered a simple and accurate method for typing *S. aureus* isolated from distinct sources[11-16]. Its discriminatory power relies on the heterogeneity of the region containing the 81 bp tandem repeats at the 3' coding region of the coagulase gene which differs both in the number of tandem repeats and the location of AluI and HaeIII restriction sites among different isolates[17].

In the present study, we have developed a typing methodology using coagulase gene polymorphisms of *S. aureus* strains obtained from various hospitals across Chennai, South India. We have also evaluated the efficiency of these typing methods in discriminating unrelated *S. aureus* strains using two restriction enzymes AluI and HaeIII for their typing ability.

## MATERIALS AND METHODS

**Bacterial strains and culture:** In this study 85 *Staphylococcus aureus* strains were used and all clinical isolates were from different clinical specimens such as pus, blood, sputum, endotracheal secretions, urine and others. Primary culture was performed on Blood agar plates. Then they were sub-cultured on Lipovitellin Salt Mannitol Agar (LSMA), a specific medium for *S. aureus*. Coagulase positive strains were identified by tube coagulase test as described earlier (coagulase test-Issue no: 3 Issue date: 07.10.05, Issued by: Standards Unit, Evaluations and Standards Laboratory).

**Disk diffusion test:** Disk diffusion test was done to distinguish MRSA and MSSA. MHA (Muller Hinton Agar) plates were inoculated with 0.5 Mc Farland standard culture, a 30 μg cefoxitin antibiotic disk was placed and incubated for 18 h at 37°C[18]. Zone diameters were measured and interpreted as MSSA (Methicillin Sensitive *Staphylococcus aureus*) or MRSA (Methicillin Resistant *Staphylococcus aureus*) according an interpretive standards guideline established by CLSI in 2007. Zone diameter ≤21 mm is taken as MRSA and ≥22 mm is taken as MSSA for coagulase positive strains. For coagulase negative *Staphylococci*, Zone diameter ≤24 mm is taken as MRSA and ≥25 mm is taken as MSSA[19,20].

**Extraction of Genomic DNA:** Genomic DNA was extracted using standard protocol[21]. Briefly, 5 mL of LB broth was inoculated with the bacterial strain and was grown overnight at 37°C. Two mL of the culture was pelleted through brief centrifugation of 15 min and resuspended in 567μL of TE buffer. 30 μL of 10% SDS and 3 μL of 20 mg $mL^{-1}$ proteinase K were added to cell suspension and incubated for 1 h at 37°C. After incubation, 100 μL of 5M NaCl and 80 μL of CTAB-NaCl were added and incubated further for 10 min at 65°C. After an addition of 0.8 mL of chloroform: Isoamyl alcohols (24:1), mixture was vortexed for 1 min. The clear supernatant was mixed with an equal volume of phenol: chloroform: Isoamyl alcohol (25:24:1) and shaken for one min. The aqueous and organic layers were separated after centrifugation for 5 min. Clear Upper layer was decanted and 0.6 vol of isopropanol was added. Resulting precipitated pellet was washed two times with 70% ethanol, air dried and finally dissolved in 100 μL of TE buffer. DNA was quantified with UV-Visible Spectrophotometer and preserved for further use.

**Polymerase Chain Reaction (PCR):** PCR amplification was performed using DNA and primers of 3'-end region of the coagulase gene and internal primers for the 3' end hyper variable region containing 81 bp tandems repeats. The sequence of forward primer used for amplification was 5'CGAGACCAAGATTCAACAAG and the reverse primer was 5'AAAGAAAACCACTCACATCA. PCR conditions used were as follows: 94°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 45 sec and 72°C for 2 min, followed by a final extension of 72°C for 7 min. Depending on the number of 81 bp repeats a strain Analysis of Restriction Fragment Length Polymorphism of PCR products performed with AluI and HaeIII. For digestion with AluI, 7 μL of PCR product was incubated with 5U of AluI in a 20 μL reaction mixture at 370°C for 16 h in a water bath. For digestion with HaeIII, 10 μL of PCR product was incubated with 6 U of the enzyme at 37°C for 1 h 45 min in a water bath[22]. The PCR products and restriction digest fragments were detected by electrophoresis through 1.6% agarose gel.

**Determination of numerical index of discrimination:** The probability that two unrelated isolates sampled from the test population will be placed into different typing groups or clusters was assessed according to the Hunter-Gaston formula[23]. This probability, also called discriminatory index is calculated using following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{J=1}^{S} n_j (n_j - 1)$$

Where:

D = Numerical index of discrimination

N = The total number of isolates in the sample population

s = The total number of types obtained

$n_j$ = The number of isolates belonging to the jth type contains

The PCR product size can range from 500-1000 bps.

## RESULTS

**Amplification of coagulase gene:** The sizes of PCR products obtained after amplification ranged from 650-1000 bps (Fig. 1). These PCR products were classified into 3 band classes based on the size (Table 1). After electrophoresis pattern analysis it was found that majority of isolates belong to the band class 2: (812 bp)

During analysis it is noted that two of the strains, classified as coagulase negative by tube coagulase test were found to be positive with PCR amplification of the gene which clearly emphasizes the use of molecular methods in detecting *Staphylococcus aureus*. Since, the Numerical index of discrimination (D-value) based on PCR product sizes were: 0.55, which is very less, typing based on PCR Product size alone cannot be used. These PCR products were further subjected to RFLP analysis with AluI and HaeIII digestion.

**AluI restriction enzyme digestion:** Restriction digestion of the PCR product with AluI gave one to five bands in each isolate (Fig. 1). Four PCR products did not show any Restriction cut with AluI. This probably indicates the absence of AluI restriction sites amongst these isolates. The bands observed by AluI digestion were in multiples of 81bp. All the bands produced by restriction digestion by AluI were divided into 9 band classes (Table 2 and Fig. 2) based on the size of the length of PCR product. Number and size of the bands were analyzed by using UPGMA (Fig. 2). Clinical isolates produced 31 distinct RFLP banding patterns with AluI and designated as A1-A31 (Table 3). The pattern A10 = '324-405' was found to be dominating since more number of isolates possess this pattern as compared to different patterns obtained from various isolates. Numerical index of discrimination (D value) was 0.96 which is described a very good index for discrimination. Discrimination index >0.90 renders RLFP analysis as an effective molecular typing tool.

Table 1: Band classes of PCR coagulase gene products

| Band class No. | Size of PCR product in base pairs |
|---|---|
| 1 | 723 |
| 2 | 812 |
| | 913 |

Cluster analysis was also performed with the Gelcompare software using the Pearson correlation coefficient to determine distance matrices and Un-weighted Pair Group Method with Arithmetic mean (UPGMA) to create dendrograms. At >80% distance similarity, AluI dendrogram (Fig. 2) was divided into 15 clusters with a cluster significance of 74. Clusters C7 and C15 are the ones having largest number of strains.

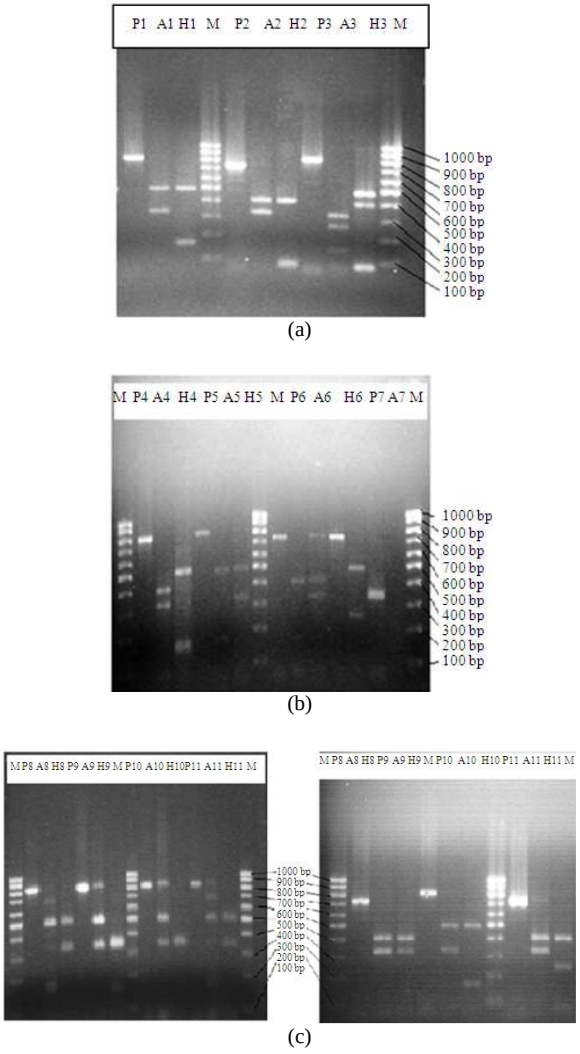


Fig. 1: Representative agarose gel electrophoresis image of coagulase gene PCR products and their corresponding AluI and HaeIII restriction enzyme digestion products, where. M: 100 bp DNA ladder; A: AluI restriction digested product; H: HaeIII restriction enzyme digestion products

Table 2: The band class by AluI restriction enzyme digestion

| Band class of AluI | Size of the fragments in base pairs |
|---|---|
| 1 | 81 |
| 2 | 162 |
| 3 | 243 |
| 4 | 324 |
| 5 | 405 |
| 6 | 486 |
| 7 | 648 |
| 8 | 729 |
| 9 | 979 |

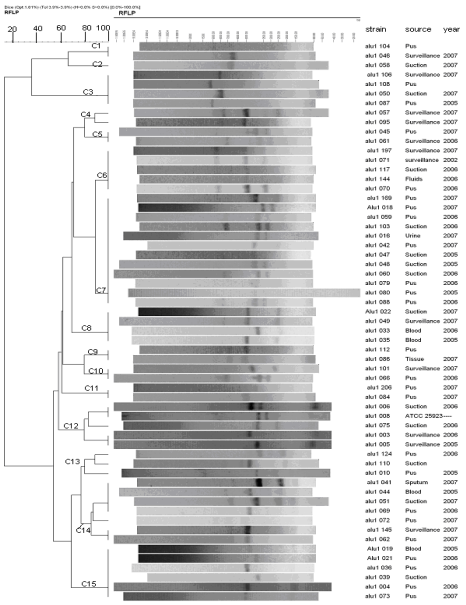


Fig. 2: Dendrogram showing the clustering of strains based on the AluI banding patterns into 15 clusters c1-c15. Also, shown here are the Strain Number, Source and year of isolation

**HaeIII restriction enzyme digestion:** Restriction digestions were performed on the amplified PCR products with HaeIII and it gave one to three bands (Fig. 1). Unlike in AluI where some of the strains were not digested, all the PCR products were digested with HaeIII and gave RFLP patterns. This indicates that all the clinical isolates genomic DNA contained HaeIII restriction sites. The bands obtained by HaeIII digestion were also observed to be multiples of 81. Bands produced by restriction digestion by HaeIII were divided into 8 band classes (Table 4). Clinical isolates produced 21 distinct RFLP banding patterns with HaeIII and were designated as B1-B21 (Table 5). Although many patterns were found, majority of the strains (53%) belong to only 2 patterns (B1 containing 25% of total isolates and B2 contains 28% of total isolates).

Table 3: Band patterns by AluI restriction enzyme digestion of PCR products

| Pattern code | Band pattern-AluI (approx bp) | Isolates (%) |
|---|---|---|
| A1 | 648 | 3.5 |
| A2 | 162-648 | 1.7 |
| A3 | 720 | 3.5 |
| A4 | 972 | 3.5 |
| A5 | 243-324-405-486-729 | 3.5 |
| A6 | 324-405-486 | 3.5 |
| A7 | 324-486 | 6.9 |
| A8 | 405-486 | 1.7 |
| A9 | 243-405 | 6.9 |
| A10 | 324-405 | 15.5 |
| A11 | 243-486 | 5.2 |
| A12 | 486-648 | 3.5 |
| A13 | 243-486-729 | 1.7 |
| A14 | 324-405-729 | 1.7 |
| A15 | 405 | 1.7 |
| A16 | 486 | 1.7 |
| A17 | 81-243-405 | 1.7 |
| A18 | 81-405-486 | 1.7 |
| A19 | 81-324-405 | 1.7 |
| A20 | 81-405 | 1.7 |
| A21 | 243-324-405-486 | 1.7 |
| A22 | 81-162-324-486-648 | 1.7 |
| A23 | 162-243-405 | 1.7 |
| A24 | 162-243-405-648 | 3.5 |
| A25 | 243-324-486-729 | 1.7 |
| A26 | 162-324-486-648 | 1.7 |
| A27 | 162-324-486-729 | 1.7 |
| A28 | 162-486-648 | 1.7 |
| A29 | 162-405 | 3.5 |
| A30 | 162-486-729 | 1.7 |
| A31 | 162-486 | 6.9 |

Table 4: The bands classes by HaeIII restriction enzyme digestion

| Band class of HaeIII | Size of the fragments in base pairs |
|---|---|
| 1 | 81 |
| 2 | 162 |
| 3 | 243 |
| 4 | 324 |
| 5 | 405 |
| 6 | 486 |
| 7 | 567 |
| 8 | 729 |

At 100% distance similarly, HaeIII dendrogram (Fig. 3) was divided into 15 clusters. Clusters H1 and H2 are having majority of the strains. For HaeIII typing, the Numerical index of discrimination (D value) was: 0.85 which was low as compared to AluI typing which had a D value of 0.96. Some of the strains from another hospital were analyzed and HaeIII digestion pattern categorized them into patterns B1 and B2 class type in which most of the strains were placed. Where as AluI typing gave unique patterns for them (A2, A16, A28) and was able to discriminate those strains from others. So AluI typing which has high discriminatory index is better than HaeIII typing when considered individually.

Table 5: Band patterns by HaeIII restriction enzyme digestion of the PCR products

| Pattern code | Band pattern-HaeIII (approx bp) | Percent of isolates |
|---|---|---|
| B1 | 324-486 | 25.0 |
| B2 | 324-405 | 28.0 |
| B3 | 243-324 | 8.0 |
| B4 | 81-324-405 | 1.7 |
| B5 | 405 | 5.0 |
| B6 | 324 | 5.0 |
| B7 | 324-405-729 | 1.7 |
| B8 | 324-720 | 1.7 |
| B9 | 486-567 | 1.7 |
| B10 | 405-486 | 1.7 |
| B11 | 383-405-567 | 1.7 |
| B12 | 243-405 | 1.7 |
| B13 | 162-405 | 1.7 |
| B14 | 162-243-324 | 1.7 |
| B15 | 81-486 | 1.7 |
| B16 | 162-567 | 1.7 |
| B17 | 162-486 | 1.7 |
| B18 | 81-162-567 | 1.7 |
| B19 | 486 | 3.3 |
| B20 | 162-324 | 1.7 |
| B21 | 81-243-324 | 1.7 |

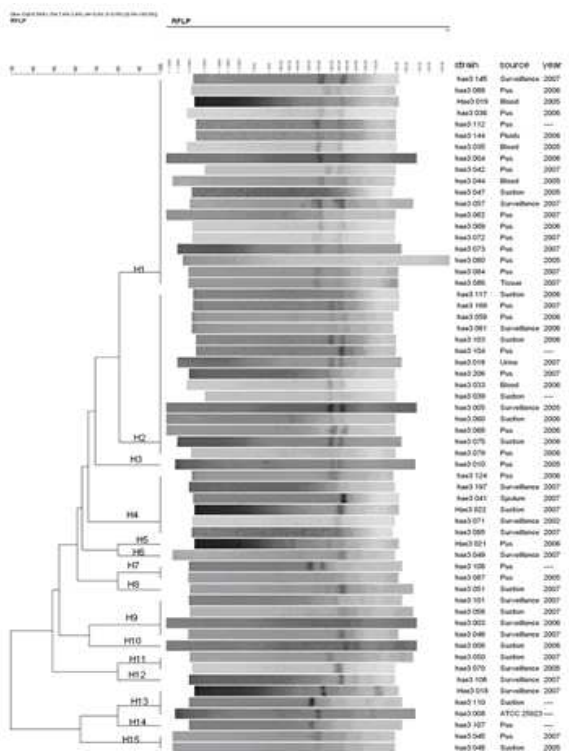


Fig. 3: Dendrogram showing the clustering of strains based on the HaeIII banding patterns into 15 clusters H1 to H15. Also, shown here are the strain number, source and year of isolation

**AluI and HaeIII combined analysis:** The combined grouping of all the RFLP increased the discriminatory index of the typing procedure. The discriminatory index of AluI and HaeIII combined classification was: 0.98 which is higher than the highest of individual typing. So it is desirable that both AluI and HaeIII should be used for better and reliable discrimination.

## DISCUSSION

Molecular strain typing of microorganisms is now recognized as an essential component of infection control program. These molecular techniques are effective in tracking the spread of nosocomial infections and planning the activities of the infection control program. Molecular typing can shorten or prevent an epidemic and reduce the number and cost of nosocomial infections[24]. If strains from two patients possess the same fingerprint, they are probably infected from the same source or transmitted from one patient to the other. This typing can also clarify whether the strains from the environment, instruments, staff, or food are responsible for causing infection. In this way this helps to trace the source of infection or an outbreak, tracking the spread of infections and helps to take specific infection control measures.

In this study, we have utilized the discriminatory power of coagulase gene typing relies on the heterogeneity of the region containing the 81 bp tandem repeats at the 3' end coding region of the coagulase gene which differs both in the number of tandem repeats and the location of AluI and HaeIII restriction sites among different isolates. The purpose of this work was to study the coagulase gene polymorphisms in clinical specimens *S. aureus* from local hospitals. We evaluated the efficiency of this typing method in discriminating unrelated *S. aureus* strains and compared two restriction enzymes AluI and HaeIII in their typing ability

The coagulase gene amplification has been considered a simple and accurate method for typing *Staphyloccus aureus*[14-16,25]. This method is found to be technically simple with a good reproducibility and discriminatory power. As comparisons between large numbers of bacterial strains can be made within a short time, present method is suitable for general clinical setting in the hospital and for epidemiological investigations of *Staphylococcus aureus.*

In some isolates, the PCR product size, RFLP patterns of surveillance strains exactly matched with that of RFLP patterns of patient's isolates. RFLP typing methodology helps to trace the source of infection and routes of transmission and thus helps to control infections and outbreaks. Also we found that no discriminative bands or banding patterns were obtained that can distinguish between MRSA and MSSA. So this

typing method cannot be used to distinguish MRSA and MSSA. Calculation of the discriminatory power of the AluI typing method yielded high DI (Discriminatory index) value: 0.96. An index greater than 0.90 can be interpreted with confidence is desirable. For HaeIII typing, DI was only 0.85 and HaeIII typing could not discriminate one hospital strains from another where as AluI typing was able to discriminate strains from different hospitals.by placing them in different band classes. AluI typing proved to be better than HaeIII typing when considered individually. No restriction sites for enzymes which would allow a greater discriminatory power than AluI were detected. This study confirmed that finding as the discriminatory index of AluI is much greater than HaeIII and it was able to discriminate unrelated strains well. The combined grouping increased the discriminatory index of the typing. The discriminatory index of AluI and HaeIII typing combined classification is: 0.98 which is higher than the highest of individual typing. So it is desirable that both AluI and HaeIII restriction enzymes are used for a better and reliable discrimination.

## CONCLUSION

With a high discriminatory index value, this method proved to be useful, technically simple, reproducible, rapid and efficient for typing *Staphylococcus aureus* strains isolated from clinical specimens. AluI is better than HaeIII in typing *Staphylococcus aureus* when considered individually but HaeIII can be used in combination with AluI for a more reliable and efficient discrimination. The information generated in this study could be useful to develop an efficient infection control measure. This typing method can also be included in a routine infection control program in hospitals and for epidemiological investigations.

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