Original Article

Molecular Genotyping of Methicillin Resistant and Susceptible *Staphylococcus aureus* by Coagulase Gene Polymorphism

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Abstract

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) which causes nosocomial infections is among the most important multi-resistant pathogens worldwide. Investigations of MRSA outbreaks in nosocomial settings often require strain-typing data to verify effectively that the isolates belong to the outbreak strain, and to discriminate similarity from unsimilarity strains. Quick and reliable typing methods are required to obtain information among MRSA isolates and to allow faster implementation of appropriate control measures.

Objective : The aim of this study was to investigate genotyping of methicillin resistant and sensitive *Staphylococcus aureus* (MRSA and MSSA) in Khartoum Teaching Hospital, Sudan by using antibiotype and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the coagulase gene.

Methods: This is across sectional study. The samples were collected from clinical wound specimens in the wards of surgery, orthopaedic and burns at Khartoum Teaching Hospital, then processed, cultured and subsequently susceptibility test was performed using disc diffusion method. The MRSA strains were investigated by oxacillin 1μg disk diffusion method. PCR used to amplify a sequence of the coagulase (*coa*) gene, and the PCR products were analyzed by PCR-RFLP using *Alu*1 restriction enzyme.

Result: Forty eight *S. aureus* strains were isolated and the number of MRSA identified was 9(18.75%). All strains of MRSA and MSSA were sensitive to vancomycin, while multi-drug resistance was common among MRSA strains. PCR amplification products of *coa* gene were approximately at 500 bp (26/48), and 580 bp (22/48). By *Alu*1 restriction enzyme digestion of the PCR-amplified of coagulase gene, two distinct PCR-RFLP patterns exhibited; *coa*A and *coa*B and their fragments were approximately at 190, 310 bp and 190, 390 bp with percentages of 54.2% (26/48) and 45.8% (22/48) respectively.

Conclusion: PCR-RFLP is considered an attractive tool for rapidly demonstrate the frequency of different patterns and discriminate the relatedness of isolates in different hospital wards.

Key words: Methicillin-resistant *Staphylococcus aureus*, coagulase gene, antibiotype, restriction fragment length polymorphism (RFLP)

*S*taphylococcus aureus has long been recognized as being an important pathogen in human disease. Serious staphylococcal infections can frequently occur in patients and may lead to dire consequences, especially for therapy with antimicrobial agents [1]. Outbreaks of hospital acquired infections due to methicillin resistant *S. aureus* (MRSA) are being reported with increasing frequency, challenging clinicians and infection control teams throughout the world[2].

Phenotypic markers can be used to identify MRSA. The antibiogram has been the main typing tool in many hospital outbreaks since the technique is widely available and standardized, and it can be used with all microbial species [3].

Coagulase production is the principal criterion used by the clinical microbiology laboratory for the identification of *S. aureus* isolates from human infections [4]. Besides the traditional typing methods for MRSA, several alternative molecular techniques have been developed for epidemiological studies.

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Among these are pulsed-field gel electrophoresis (PFGE)[3], polymerase chain reaction (PCR) of the coagulase (*coa*) gene, the protein A (*spa*) gene, hypervariable regions (HVR) adjacent to the *mec*A gene [5,6.] DNA sequencing and polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) of the *coa* and *spa* genes [7]. The PCR amplifications of these particular regions produce DNA fragments of different sizes and are highly polymorphic with regard to the number and sequence of the repeats [8].

Investigation of staphylococcal isolates (MRSA and MSSA) outbreak in nosocomial settings often requires strain-typing data to verify effectively that the isolates belong to the outbreak and discriminate similarity from unsimilarity strains. Therefore, the aim of this study was to investigate the epidemiological typing of *S. aureus* isolates from Khartoum Teaching Hospital by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the coagulase (*coa*) gene.

Materials and Methods

The samples were collected from clinical wound specimens in the wards of surgery, orthopaedic and burns at Khartoum Teaching Hospital, Sudan in the period September 2005 to August 2007. *S. aureus* ATCC 43300, coagulase negative staphylococci (*S. epidermidis*), were included as positive and negative controls for PCR testing of the *coa* gene polymorphism.

All isolates were identified by standard microbiological methods including Gram stain, catalase, coagulase, DNase and growth on mannitol salt agar. Resistant to oxacillin was determined by the disc diffusion method according to the National Committee for Clinical Laboratory Standard (NCCLS) guidelines.

Antimicrobial susceptibility testing was performed by the disc diffusion method on Mueller-Hinton agar (Oxoid), to tested 10 antimicrobial agents: cephalexine Cp (30 mcg), co-trimoxazole Co (25 mcg), clindamycin Cd (2 mcg), erythromycin E (15 mcg), vancomycin Va (30 mcg), tetracycline T (30 mcg), rifampicin R (5 mcg), amoxicillin Am (10 mcg), and ciprofloxacin Cf (5 µg). A suspension of the tested organisms was adjusted against 0.5 MacFarland standard turbidity and inoculated onto media, then incubated at 35-37 °C for 16-18 hours, and examined for evidence of growth.

Stock clinical cultures were maintained in brain heart infusion broth-glycerol mixture (3:1 vol/vol) at –70ºC until further molecular analysis [2].

Genomic DNA was isolated by using chloroform DNA purification protocol. With a 1µl loop, a small quantity of growth equivalent to about two small colonies was scraped from the top of the culture and placed into 100µl of sterile distilled water in a microcentrifuge tube. Chloroform 100µl (Sigma) was added, and the mixture was vortexed for about 10 seconds. The mixture was heated at 80°C for 20 minutes, after which time it was held at -20°C for at least 20 minutes. The sample was allowed to thaw but while still cold was centrifuged at 12,000xg for 3 minutes in a minicentrifuge. The DNA was stored at -20°C and ready for use[9].

The *coa* gene was amplified with two primers, as described by [8]. The forward primer 5′-ATA GAG ATG CTG GTA CAG G-3′ (1513 to 1531) and the reverse primer 5′-GCT TCC GAT TGT TCG ATG C-3′ (2188 to 2168) were chosen. The PCR reaction mixture contained of reaction buffer (5µl), 500U Go Taq DNA polymerase (0.5µl) (Promega), 10mM dNTPs mix (1µl), 25mM $MgCl_2$ (3µl), 10PM forward primer (12µl), 10PM reverse primer (8.8µl), template DNA (5µl), and distilled water (14.7µl) to a final volume 50µl. DNA amplifications were performed in thermal cycles as the following cycling parameters: an initial step at 94°C for 3 minutes; 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and a final step at 72°C for 4 minutes. The PCR-amplified samples were subjected to electrophoresis on 0.8% agarose gel with ethidium bromide incorporated for DNA staining. The PCR products were visualized and photographed on an UV transilluminator,

and the sizes of the PCR products were determined by comparison to 100-1000 ladder DNA marker (Sigma).

After amplification, ten microliters of PCR product was mixed and digested with 2 units (0.2μl) of restriction endonuclease *Alu*I (Promega) at 37°C for 1 hr 30 minutes. The restriction digested fragments were electrophoresis on 1% agarose gel, which were visualized under UV light. Photographs of agarose gel were taken using gel documentation system, and the pictures were further analyzed with computer and printed.

Results

Forty eight *S. aureus* isolates were collected and the number of MRSA identified was 9(18.75%). All isolates were classified into 3 groups; group I: *S. aureus* isolated from surgical ward (No. = 28; 58.3%), group II: *S. aureus* isolated from orthopaedic ward (No. = 14; 29.2%), and group III: *S. aureus* isolated from burns unit (No. = 6; 12.5%).

The susceptibility test was conducted for 48 *S. aureus* isolates against 10 antimicrobial agents by disc diffusion method. All isolates showed 100% sensitivity to vancomycin. For the other antibiotics, the degree of sensitivity was; co-trimoxazole 27 (56.25%), rifampicin 35 (72.9%), clindamycin 33 (68.75%), tetracycline 16 (33.3%), erythromycin 31 (64.6%), cephalexine 29 (60.4%), methicillin 39 (81.25%), amoxicillin 19 (39.6%), and ciprofloxacin 32 (66.7%).

All 48 *S. aureus* isolates examined produced PCR amplicon when coagulase gene primers were used. The two PCR size products obtained were approximately at 500 bp (26 isolates), and 580 bp (22 isolates). An isolate of CoNS (*S. epidermidis*) and *Escherichia coli* which served as negative controls produced no DNA product following PCR amplification (Fig. 1).

PCR products from coagulase gene specific primers were digested with *Alu*I restriction enzyme, and the resulting fragments were separated by gel electrophoresis. Two distinct patterns were detected among *S. aureus* isolates; *coa*A and *coa*B with sizes

approximately at 190, 310 bp and 190, 390 bp. The percentages of these patterns were 54.2% and 45.8% respectively (Fig. 2).
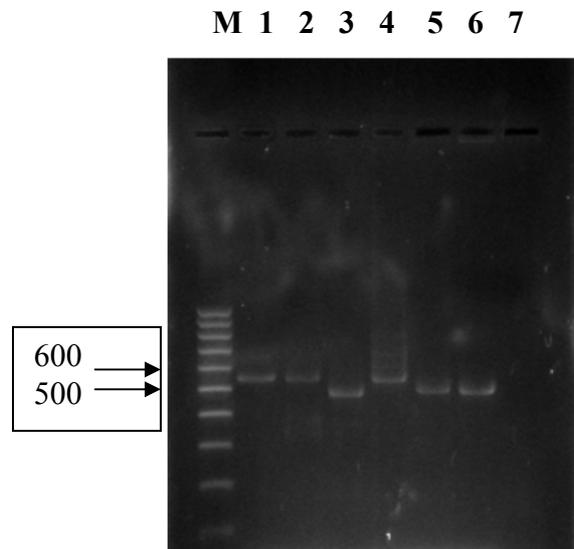

Figure 1: 0.8% agarose gel electrophoresis of PCR amplification of the coagulase gene products from the *S. aureus* isolates. Lane M (100 bp ladder) DNA marker. Lanes 1, 2, 4 580 bp fragment. Lanes 3, 5, 6 500 bp fragment. Lane 7 no DNA (CoNS) *S. epidermidis* product.
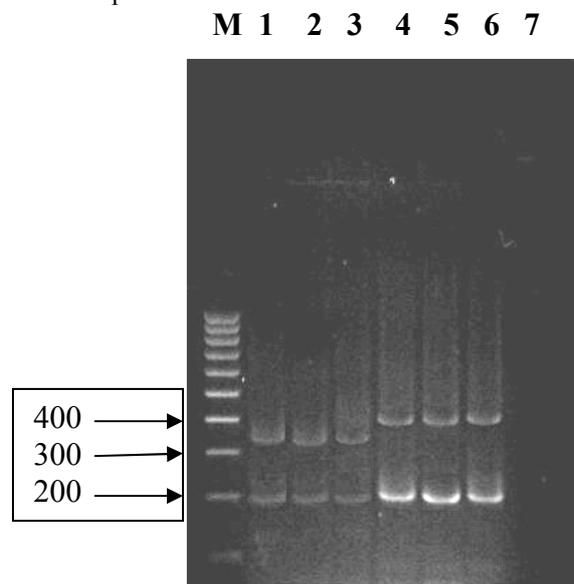

Figure 2: 1% agarose gel electrophoresis of *Alu*I restriction enzyme digestion products of the coagulase gene PCR products. Lane M (100 bp ladder) DNA marker. Lanes 1-3 pattern *coa*A (190 and 310 bp). Lanes 4-6 pattern *coa*B (190 and 390 bp).

The frequency of different patterns in different hospital wards as following; group I surgical ward: the prevalence of pattern A was 15 of 28 (53.6 %) and pattern B was 13

of 28 (46.4 %), group II orthopaedic ward: the prevalence of pattern A was 9 of 14 (64.3 %) and pattern B was 5 of 14 (35.7 %), group III

burns unit: the prevalence of pattern A was 2 of 6 (33.3 %) and pattern B was 4 of 6 (66.7 %) (Table 1).

Table 1: Frequency of *S. aureus* /PCR-RFLP in each hospital wards

coa patterns	Size of *Alu*I restriction digest fragments (approximately bp)	Total (No. 48) No. (%)	Surgery (No. 28) No. (%)	Orthopedic (No. 14) No. (%)	Burns (No. 6) No. (%)
A	190-310	26 (54.2)	15 (53.6)	9 (64.3)	2 (33.3)
B	190-390	22 (45.8)	13 (46.4)	5 (35.7)	4 (66.7)
Total		48 (100)	28 (100)	14 (100)	6 (100)

Discussion

The objective of this study was to determine whether PCR-RFLP of the coagulase gene could be used to differentiate the similarity or unsimilarity between the tested isolates. The coagulase gene from 48 isolates was amplified by PCR, and the products were digested with endonuclease enzyme *Alu*I. Therefore, PCR-RFLP analysis showed that two different restriction profile patterns *coa*A, and *coa*B, could be identified according to their PCR end products at 190, 310 bp and 190, 390 bp respectively. The use of DNA restriction endonuclease to digest the coagulase gene was beneficial in confirming the two distinct RFLP patterns among *S. aureus* strains. In addition, PCR-RFLP pattern analysis allowed the differentiation of isolates (Fig. 2). The prevalence of different patterns in different hospital wards could be demonstrated, as well as the similarity or unsimilarity between the tested isolates.

Coagulase is produced by all strains of *S. aureus,* its production is the principal criterion used in the clinical microbiology laboratory for the identification of *S. aureus* in human infections, and it is thought to be an important virulence factor [10]. The *coa* gene coding for the coagulase protein has been considered a candidate for the development of DNA-based diagnostic assays for *S. aureus*. We presented evidence of existence type of coagulase gene among 48 *S. aureus* strains

examined by PCR, and the products generated size variations in the amplicon primer. We found two size products obtained approximately at 500 bp and 580 bp of PCR-coagulase gene, because this gene is highly polymorphic.

Numerous allelic forms of *S. aureus* coagulase exist, with each isolate producing one or more of these enzyme variants. By digestion with *Alu*I restriction enzyme of the amplified gene product, it is possible to discriminate between *S. aureus* isolates by RFLP of the coagulase gene [4]. Therefore, the main purpose of present study was to establish a rapid and reliable molecular typing method for *S. aureus* by means of PCR-RFLP of coagulase gene, and this was accomplished within a single PCR-coagulase gene following amplimer digestion with *Alu*I that resulted in reproducible restriction patterns and allowed sufficient discrimination among strains isolated from Khartoum Teaching Hospital.

For RFLP coagulase gene typing, two distinct patterns were detected, and the number of fragments produced upon *Alu*I digestion were two bands. These results differ from previous studies in which *Alu*I PCR-RFLP fragments varied from one to four bands using the same primer sequences [8, 10]. However, other studies with different primer sequence of *coa* gene showed *Alu*I PCR-RFLP fragments varied from two to three bands [4, 11].

In this study we found that the *coaA* pattern was common in multiple antibiotic resistance strains MRSA and MSSA, and the DNA sequence differences in the staphylocoagulase gene are responsible for the polymorphic restriction fragment length patterns seen among the *S. aureus* isolates. The reason for this polymorphism in the coagulase gene among *S. aureus* strains is unclear, but the extensive polymorphism observed does suggest that the coagulase gene may be an important virulence determinant for this organism [4].

The coagulase gene RFLP method for typing of *S. aureus* isolates is far simpler than those previously reported. It requires only small quantities of crude DNA, and individual strains can be compared easily by both the number of PCR-amplified gene products and the sizes of their *Alu*I restriction enzyme digest fragments. However, the coagulase gene typing has been reported to be a new attractive method for clinical laboratories because of its ease and speed[4].

Conclusion

Restriction analysis by PCR-RFLP permitted discrimination of the tested strains by exhibiting specific PCR-RFLP patterns, and we could rapidly demonstrate the prevalence of different patterns in different hospital wards between the tested isolates. Therefore, PCR-RFLP represents a powerful, rapid and reliable molecular typing system as well as an alternative tool for routine epidemiological surveillance and infection control measures.

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