Molecular typing of methicillin resistant *Staphylococcus aureus* by spa gene polymorphism

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The aim of this study was to detect different genotypes of methicillin resistant *Staphylococcus aureus* (MRSA) by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the spa gene in Cairo University hospital. A total of 48 samples were obtained from the sputum, infected wound, blood and pleural fluid from Cairo University hospital from the main microbiology laboratory for three month duration, they were then processed, cultured and subsequently susceptibility test was performed using disc diffusion method. The strains were investigated by cefoxitin 30 μg disk diffusion method. PCR was used to detect mec A gene, and to amplify sequence of spa gene, and the PCR products were analyzed by PCR-RFLP using HaeIII restriction enzyme. A total of 48 samples were obtained from the sputum, infected wound, blood and pleural fluid from Cairo University hospital from the main microbiology laboratory for three month duration. 37 (77%) isolates were resistant to cefoxitin, all tested isolates gave PCR positive for mec A gene, PCR-RFLP of the spa gene showed 18 (37.5%) isolates with pattern I, one of them showed pattern I, II, IV together. 8 (16.6%) isolates showed pattern II, one of them showed pattern I, II, IV; 9 (18.7%) isolates showed pattern III, 3 (6.2%) isolates showed pattern IV; one of them showed pattern I, II, IV; 8 (16.6%) isolates showed pattern V. 2 (4%) isolates were negative, and 1 isolate showed bands at 243, 667, 715 and 811 bp, 1 isolate showed bands at 243, 264 and 739 bp. PCR-RFLP typing method is a good practical tool in routine epidemiological surveillance. It's easy in performance and interpretation, and it can replace PFGE which is time-consuming and expensive.

Key words: Methicillin-resistant *Staphylococcus aureus*, mecA gene, spa gene, restriction fragment length polymorphism (RFLP).

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) which causes noscomial infections, is among the most important multi-resistant pathogens worldwide (Tambic et al., 1997). A variety of typing techniques have been developed to discriminate the related strains from unrelated strains. Accurate and rapid typing is crucial for the control of MRSA outbreaks (Maslow and Mulligan, 1996). Both phenotypic and genotypic characterizations can be used to identify epidemic MRSA (Janwithayanuchit et al., 2006). Cephamycins were used extensively in Japan in the early 1980s, and as a result some methicillin resistant *Staphylococcus epidermidis* (MRSE) and MRSA isolates became resistant to cefoxitin. Surprisingly, cefoxitin induced production of PBP2a in vitro in MRSA isolates for which cefoxitin MICs were high, and the disk diffusion assay with cefoxitin proved to be a good assay for detection of low-level MRSA in Japan (Mervat and Fateen, 2004).

Felten et al. (2002) found that, the cefoxitin and moxalactam disk diffusion method was very suitable for detection of MRSA. With the low-density inoculum (10^6 CFU/ml) at 37°C, all MRSA isolates showed cefoxitin inhibition zone diameters of <27 mm and moxalactam inhibition zone diameters of <24 mm, and all MSSA isolates showed larger diameters. With these critical diameters, cefoxitin and moxalactam disk diffusion tests were 100% sensitive and 100% specific. Nearly all MRSA isolates produce an additional penicillin-binding protein (PBP), named PBP2a. PBP2a binds B-lactams with a lower affinity than PBP2, the major physiological methicillin target. PBP2a is encoded by the mecA gene, a component of a larger DNA fragment designated the mec region. The standard test used to identify MRSA is amplification of the mecA gene (Felten et al., 2002).
Strategies aimed at preventing the spread of MRSA require a thorough knowledge of both the dissemination and the epidemiology of MRSA strains. For this purpose, various molecular typing techniques have been developed.

These techniques include pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), SCCmec typing, and typing of the variable tandem repeat region of protein A (spa typing) (Deurenberg et al., 2006). The spa gene (protein A), respectively, could be used for reliable and accurate typing of MRSA (Harmsen et al., 2003).

The aim of this study was to detect different genotypes of methicillin resistant *S. aureus* (MRSA), by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the spa gene in Cairo University hospital.

**MATERIALS AND METHODS**

**Isolation and identification of *S. aureus***

A total of 48 samples were obtained from the sputum, infected wound, blood and pleural fluid from Cairo University hospital from the main microbiology laboratory for three months duration. No duplicate isolates from a single patient were included in this study. Bacteria were grown overnight on blood agar plate at 37°C, in an aerobic atmosphere and standard microbiological methods for identification were done including Gram staining, mannitol fermentation on mannitol salt agar, catalase, DNase, slide and tube coagulase (Brown et al., 2005). The antibiotic sensitivity profile of the 48 *S. aureus* isolates were determined according to the method of kibrybauer (Bauer et al., 1966) using discs of antibiotics places on the surface of Mueller Hinton agar medium seeded with the test organism. Inhibition zones were measured after 24 h of incubation at 37°C. Interpretation of resistance was based on CLSI, 2011. The antibiotics used were ciprofloxacin, clindamycin, erythromycin, rifampcin, trimethoprim/sulfamethoxazole, cephalozin, doxycycline, gentamicin, cefoxitin and vancomycin (Oxoid-England).

**Detection of methicillin resistance**

Methicillin resistance was determined for all the *S. aureus* isolates by disc diffusion method using cefoxitin disc, according to the guidelines of the Clinical Laboratory Standard Institute (CLSI, 2011). The plates were incubated in ambient air at 35°C for 24 h. Any growth with ≤21 mm in diameter zone around the disk was considered indicative of resistance. *S. aureus* colonies were preserved in glycerol broth at -70°C until further molecular analysis. Methicillin resistance was further confirmed by the detection of *mecA* gene (encoding high resistance to methicillin) by the PCR method, which is described below:

**DNA isolation and PCR**

DNA was extracted from *S. aureus* colonies using the Quiagen DNA Mini kit51304). From this suspension, a 5 μL volume was directly used as template for PCR amplification of *mecA* and spa gene fragments. *mecA1* (5' - GTA GAA ACT AGT GAA CGT CCG ATA A - 3') and *mecA2* (5' - GCT ATT CCA CAT TGT TTT GGT CTA A - 3') primers were used for the amplification of the 310 bp fragment of the methicillin-resistant gene (*mecA*) (Perez-Roth et al., 2001). Positive control was used for *S. aureus* ATCC 43300. Also, negative control was used and primers spa1 (5'- ATC TGG TGG CGT ACC TG - 3') and spa2 (5' - CGC TGC ACC TAA CGG TAA TG - 3') were used to amplify the portion of the spa gene (Wichelhaus et al., 2001).

**Amplification**

The PCR mixture consisted of master mix (Fermentas), 1 μL of primers and 5 μL of DNA extract in a final volume of 25 μL. For spa gene segment amplification, the PCR conditions were as follows in Parkin Elmer 9700 thermocycler: According to (Mehndiratta et al., 2009) initial denaturation at 94°C for 7 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 5 min. According to Mehndiratta et al. (2009), the part of the *mecA* gene was amplified under similar conditions except that the annealing temperature was 60°C and extension was for 40 s. The PCR products were subjected to 2% agarose gel electrophoresis.

**PCR-RFLP**

RFLP of the PCR product was carried out by the modified method of Wichelhaus et al. (2001). But I used 7μ DNA product, 2 μ buffer and 1μ of Haell restriction enzyme (Fastdigest Fermentas) which is the isoschizomers of *Bsp* 143II restriction enzyme) at 37°C for 5 min. Digested fragments were separated on 3% agarose gel. While Mehndiratta et al. in 2009 used *Bsp* 143II restriction enzyme.

**RESULTS**

Out of the seven isolates of sputum, 34 isolates of pus, 6 isolates of blood, 1 isolate of pleural fluid (Table 1), 37 (77%) isolates were resistant to cefoxitin. 11 (22.9%) isolates were sensitive to cefoxitin. Regarding PCR analysis, detection of *mecA* gene for all tested isolates gave PCR end product at 310 bp as shown in Figure 1. The bands were photographed using gel documentation system.

**PCR-RFLP of the spa gene**

These PCR products, after digestion with *Hae* II restriction enzyme, showed five distinct *spa* banding patterns. This classification is according to Mehndiratta et al. (2009):

Pattern I: 243 bp, 438 bp, 715 bp
Pattern II: 243 bp, 264 bp, 667 bp
Pattern III: 243 bp, 264 bp, 643 bp
Pattern IV: 243 bp, 264 bp, 811 bp
Pattern V: 243 bp, 438 bp, 739 bp

18 (37.5%) isolates showed pattern I; one of them showed patterns I, II and IV. 8 (16.6%) isolates showed pattern II; one of them showed patterns I, II and IV, 9 (18.7%) isolates showed pattern III; 3 (6.2%) isolates showed pattern IV; one of them showed patterns I, II, IV; 8 (16.6%) isolates showed pattern V. 2 (4%) isolates showed negative, and 1 isolate showed bands at 243, 667, 715 and 811 bp, 1 isolate showed bands at 243, 264 and 739 bp (Figures 2 and 3).
Table 1. Sensitivity pattern of MRSA by disc diffusion method.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Number of sensitive isolate</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin</td>
<td>48/48</td>
<td>100</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>34/48</td>
<td>70.8</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>35/48</td>
<td>72.9</td>
</tr>
<tr>
<td>Trimethoprim/Sulfamethoxazole</td>
<td>46/48</td>
<td>95.8</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>17/48</td>
<td>35.4</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>14/48</td>
<td>29.1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>26/48</td>
<td>54.1</td>
</tr>
<tr>
<td>Rifampin</td>
<td>44/48</td>
<td>91.6</td>
</tr>
<tr>
<td>Cephalosporin</td>
<td>0/48</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 1. PCR analysis, detection of mecA gene, product at 310 bp; ladder was used with bands 50, 100, 150, 200, 250, 300, 400, 500. Lane 1 is the positive control, lane 13 is the negative control.

DISCUSSION

Methicillin-resistant Staphylococcus aureus (MRSA) have become increasingly prevalent worldwide. Rapid and accurate identification of MRSA is required to help clinicians select appropriate antibiotic treatment and to avoid the spread of these strains (Skov et al., 2003).

Routine oxacillin tests often fail to detect very heterogeneous MRSA populations, which consequently are considered methicillin-susceptible *S. aureus* (MSSA) because of their usual susceptibility to most non-β-lactam antistaphylococcal antibiotics. The cephapmycin disk test (cefoxitin) is an available alternative to the oxacillin disk method for routine antibiotic susceptibility testing at 37°C (Felten et al., 2002). According to their results, the cefoxitin and moxalactam disk diffusion tests detected 100% of all the MRSA classes. An *S. aureus* isolate that gives a cefoxitin diameter of <27 mm or a moxalactam diameter of <24 mm can be identified as MRSA. In 2006, Skov and his coworkers suggested MRSA interpretive
criteria of susceptible S ≤ 4 mg/L and R > 4 mg/liter, corresponding to S ≥ 22 mm and R ≤ 21 mm for the 30-μg disk and S ≥ 17 mm and R ≤ 16 mm for the 10-μg cefoxitin disk.

However, there is no optimal phenotypic method for detecting methicillin resistance in S. aureus and genotypic tests involve mecA gene detection by PCR, which are considered to be the reference (Boutiba-Ben Boubaker et al., 2004).

According to Felten and his colleagues in 2002, detection of the mecA gene by PCR was considered to be the “gold standard.” In our study, PCR for mecA gene was much more helpful than cefoxitin disk. In a study done by Jain et al. in 2008, the cefoxitin disc diffusion test could detect methicillin resistance in 68/73 (93.1%) mecA-positive S. aureus while in our study, 37/48 (77%) isolates were resistant to cefoxitin.

In addition to the use of spa typing for outbreak investigation, spa typing may prove useful as a practical method for describing a natural population of S. aureus organisms. This may aid in the identification of strains that have special virulence properties or drug resistance since in many bacteria these are believed to be non randomly distributed along clonal lines (Shopsin et al., 1999). Pulsed field gel electrophoresis still remains the gold standard for MRSA typing, in spite of it being a lengthy and elaborate technique. Ways to effectively enhance its reproducibility, especially its inter-laboratory reproducibility, would further validate it as the technique of choice (Trindade et al., 2003).

According to Strande’n and his coworkers in 2003, pulsed-field gel electrophoresis (PFGE) is considered the “gold standard” for molecular typing of MRSA. However, the method is time-consuming and expensive, and its discriminatory power may not be necessary in outbreak situations

According to Wichelhaus and his colleagues in 2001, the new and rapid PCR-RFLP typing method is an attractive tool in routine epidemiological surveillance. Its impressive characteristics are ease of performance and interpretation, while at the same time guaranteeing good discriminatory power, reproducibility and typeability.

In a study that was done by Mehndiratta and his colleagues in 2009, molecular typing of MRSA based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of spa gene was done. Among 125 isolates, pattern I was most common (34.4%) followed by pattern II (24.8%), pattern V (17.6%), pattern IV (13.6%) and pattern III (9.6%), respectively. While in our study, 18 (37.5%) isolates showed pattern I, 8 (16.6%) isolates showed pattern II, 9 (18.7%) isolates showed pattern III, 3 (6.2%) isolates showed pattern IV, 8 (16.6%) isolates showed pattern V. (Figures 2 and 3). So in our study, pattern I is the most common followed by pattern III, then pattern II, V, and then pattern IV.

Conclusion

PCR-RFLP typing method is a good practical tool in routine epidemiological surveillance. It is easy in performance and interpretation, and it can replace PFGE which is time-consuming and expensive.

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