Original Article

Antibiotic Resistance Genes Detected by Multiplex PCR Assays in Staphylococcus epidermidis Strains Isolated from Dialysis Fluid and Needles in a Dialysis Service

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SUMMARY: The rate of the onset of methicillin-resistant Staphylococcus epidermidis infections is increasing in Tunisia. We have isolated 32 S. epidermidis strains from dialysis fluid and needle cultures in dialysis service. The strains were identified by classic methods (colonial morphology, Gram staining, catalase test, coagulase test, and DNase test) as well as by API ID32 Staph. Susceptibilities to 18 antibiotics were tested with the ATB Staph kit. Most of the tested strains were resistant to penicillin. In addition, the presence of multidrug resistant strains that showed resistance to different antibiotics was recorded. We have characterized these strains by multiplex PCR assay to identify intercellular adhesion genes icaA/icaD associated with the adhesiveness of staphylococci in biomaterials, and to identify representative resistant genes: oxacillin resistance, meca; erythromycin methylase (ermA, ermB, and ermC), and macrolide efflux gene (msrA and mef). The frequency of the carriage of these genes was icaA/icaD (71.9%), meca (78.1%), ermA (12.5%), ermB (31.3%), ermC (53.1%), msrA (68.8%), and mef (0%). Although the carriage of the genes and the results of susceptibility testing did not match exactly, it could be judged that the PCR identification of antibiotic resistance genes is rapid and supplementary methods for identifying staphylococci or epidemiological study used for the control of nosocomial infection.

INTRODUCTION

Staphylococcus epidermidis is the most common etiologic agent of diseases like bacteremia, osteomyelitis, urinary tract infections, and peritonitis caused by ambulatory dialysis, with a frequent association with colonization of intravascular catheters and orthopedic devices (1,2). Methicillin-resistant staphylococci are resistant to all penicillins, penems, carbapenems, and cephalosporins (3). Erythromycin resistance in Gram-positive bacteria is generally mediated by one of three mechanisms: target site modification, enzymatic inactivation of the drug, or active efflux of the antibiotic from the cell (4,5). Molecular genetic studies have produced a great deal of genetic information that can be used for diagnosis of antimicrobial resistance determinants. Cross-resistance to macrolides, lincosamides, and type B streptogramins (MLSb phenotype) is the most common, efficient, and widespread mechanism of resistance to macrolide antibiotics (6). Resistance to erythromycin in staphylococci is usually associated with resistance to other macrolides. Three genes (ermA, ermB, and ermC) encoding methylases have been found in staphylococci (7,8). Another mechanism of inducible resistance to erythromycin is conferred by the msrA gene, which encodes an ATP-dependent efflux pump (9). As a member of this family, msrA confers inducible resistance to erythromycin and type B streptogramins in staphylococci (10). The msrA resistance determinant has been found responsible for erythromycin resistance in 36.4% of clinical coagulase-negative staphylococci (CoNS) (7). On the other hand, the macrolide efflux is affected by a membrane protein encoded by the mef gene (4).

The objective of this study was to determine the antibiotic susceptibility of 32 S. epidermidis strains isolated from dialysis fluids and needles culture. The presence of intercellular adhesion gene (icaA and icaD) and the oxacillin resistance gene (meca) were detected by polymerase chain reaction (PCR) in order to examine the genetic mechanisms of drug resistance. In addition, the erythromycin resistance genes (ermA, ermB, and ermC) and the macrolide efflux (msrA and mef) were determined by PCR multiplex assays.

MATERIALS AND METHODS

Bacterial strains and biochemical tests: Dialysis water samples and needles were obtained from a dialysis service in Kairouan (Centre of Tunisia). Dialysat samples were collected every 15 days from February to November 2004. Needles were aseptically removed and analyzed from patients after 4 h of initiation of dialysis sitting. Thirty-two S. epidermidis strains were finally recovered by this procedure. The API ID32 Staph system (bio-Merieux, Ltd., Marcy l’Etoile, France) was used for identification and characterization of isolated strains according to the manufacturer’s instructions.

Antimicrobial susceptibility testing: The antimicrobial susceptibility of the 32 strains was analyzed using the ATB Staph strips (bio-Merieux) which contained a selection of 18 antibiotics, and the results were interpreted with an automate mini-Api (bio-Merieux) according to the published guide-
lines of the manufacturer.

Molecular typing: PCR of icaA and icaD gene: Chromosomal DNA was extracted using a Wizard genomic purification kit (Promega, Madison, Wis., USA) and 60 µl of lysozyme at 0.1 mg/ml (Sigma, St. Louis, Mo., USA) was added to the cell lysate. Negative PCR control without the bacterial DNA was included with each PCR reaction. The icaA and icaD gene responsible for slime production were detected by PCR using forward and reverse primers, as described previously (11). The PCR was performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, Calif., USA). PCR products (10 µl) were analyzed on 2% agarose gel stained with ethidium bromide (0.5 µg/ml), which was visualized under UV transillumination and photographed using a Gel Doc XR apparatus (Bio-Rad, Herceules, Calif., USA).

PCR for the detection of the mecA gene: The resistance to the oxacillin obtained by the ATB Staph system was confirmed by PCR of the mecA gene, as described previously (12). The PCR was performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, Calif., USA). PCR products (10 µl) were analyzed on 2% agarose gel stained with ethidium bromide (0.5 µg/ml), which was visualized under UV transillumination and photographed using a Gel Doc XR apparatus (Bio-Rad, Herceules, Calif., USA).

Biochemical characterization and antibiotic susceptibility: Thirty-two S. epidermidis strains were isolated and identified by API ID 32 Staph. The antibiotic susceptibility test carried out using an ATB Staph strips revealed the presence of multi-resistant strains towards the 18 previously cited antibiotics. The strains showed 93.8% resistance in the case of penicillin to as low as 15.6% for gentamycin, with a different extent of resistance to the other different antibiotics. On the other hand, the resistance to the other antibiotics was as follows: tetracycline (68.7%), kanamycin (53.2%), fusidic acid (46.9%), lincomycin (40.6%), pefloxacin (37.6%), oxacillin (37.5%), erythromycin (34.4%), teicoplanin (31.3%), cotrimoxazol (28.2%), fosfomycin (28.1%), tobramycin (21.9%), and rifampicin (21.9%). However we noted that all tested strains were susceptible to minocyclin, pristinamycin, nitrofurantoin, and vancomycin.

Detection by PCR of the intercellular adhesion gene icaA and icaD: Among the 32 S. epidermidis tested by PCR, 23 were both icaA and icaD positive and the nine remaining strains were both icaA and icaD negative. As shown in Fig. 1, all strains that were positive for icaA were also positive for icaD, giving 187-bp and 197-bp bands for the icaA and icaD, respectively.

Detection of resistant genes by PCR: mecA positive strains generated a DNA fragment of 310 bp by PCR (Fig. 2). In the present study, 25 of the 32 (78.1%) strains were mecA positive. The majority of mecA positive strains were isolated from needle cultures. Erythromycin-resistance genes were identified by two multiplex PCRs, one for identifying ermA, ermC, and msrA genes, and the other for identifying ermB and mef genes. Three genes encoding ribosomal methylase

Table 1. List of primer used for the detection of genes encoding antibiotic resistance

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer 5’-3’</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>icaA</td>
<td>5´-TCT CTT GCA GGA GCA ATC AA-3´</td>
<td>187</td>
<td>Anciola et al. (11)</td>
</tr>
<tr>
<td>icaD</td>
<td>5´-ATG GTA AAG CCC AGA CAG AG-3´</td>
<td>197</td>
<td>Anciola et al. (11)</td>
</tr>
<tr>
<td>mecA</td>
<td>5´-AAC AGG TGA ATT ATG AGC TGT AAG-3´</td>
<td>310</td>
<td>Ghez et al. (12)</td>
</tr>
<tr>
<td>ermA</td>
<td>5´-TAT CTT ATG GTT GAG AGG GTT-3´</td>
<td>139</td>
<td>Martineau et al. (13)</td>
</tr>
<tr>
<td>ermB</td>
<td>5´-CTA CAC TTC GCT GAT GAA AAA-3´</td>
<td>142</td>
<td>Martineau et al. (13)</td>
</tr>
<tr>
<td>ermC</td>
<td>5´-CTT GTT GAT CAC GTA AAT TTC C-3´</td>
<td>190</td>
<td>Martineau et al. (13)</td>
</tr>
<tr>
<td>msrA</td>
<td>5´-TCC ATG AGC ACA AAA TC-3´</td>
<td>163</td>
<td>Martineau et al. (13)</td>
</tr>
<tr>
<td>mef</td>
<td>5´-AGTACATTATCAGTACGC-3´</td>
<td>348</td>
<td>Lim et al. (14)</td>
</tr>
</tbody>
</table>

RESULTS

Biochemical characterization and antibiotic susceptibility: Thirty-two S. epidermidis strains were isolated and identified by API ID 32 Staph. The antibiotic susceptibility test carried out using an ATB Staph strips revealed the presence of multi-resistant strains towards the 18 previously cited antibiotics. The strains showed 93.8% resistance in the case of penicillin to as low as 15.6% for gentamycin, with a different extent of resistance to the other different antibiotics. On the other hand, the resistance to the other antibiotics was as follows: tetracycline (68.7%), kanamycin (53.2%), fusidic acid (46.9%), lincomycin (40.6%), pefloxacin (37.6%), oxacillin (37.5%), erythromycin (34.4%), teicoplanin (31.3%), cotrimoxazol (28.2%), fosfomycin (28.1%), tobramycin (21.9%), and rifampicin (21.9%). However we noted that all tested strains were susceptible to minocyclin, pristinamycin, nitrofurantoin, and vancomycin.

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genes, *ermA, ermB*, and *ermC*, were identified. *ermA* gene was present in 4 of 32 strains (12.5%). Ten strains carried the *ermB* gene and 17 of 32 (53.1%) were positive for *ermC* (Fig. 3). The *msrA* gene was identified in 22 of 32 strains (68.8%), whereas, the *mef* gene was not identified in all tested strains. In 5 strains (E7, E20, S23, S27, and S40), the *ermA* and *ermC* gene were not detected, although they were resistant to erythromycin (Table 3).

**Correlation between PCR and susceptibility testing:** As shown in Table 2, 16 strains of *S. epidermidis* were susceptible to oxacillin but they carry the *mecA* gene. Nine strains were oxacillin-resistant and they contain the *mecA* gene. In addition, 11 strains were positive for *ermC* but susceptible to erythromycin. Furthermore, only 6 strains (21.87%) were both susceptible to erythromycin and did not contain any erythromycin resistance gene, as presented in Table 3. The results show that 8 of 12 oxacillin-resistant strains were erythromycin-resistant and contain at least 1 erythromycin resistance gene.

**DISCUSSION**

In this study, 32 *S. epidermidis* strains were isolated and identified from dialysis fluid and needles culture. The antibiotic susceptibility revealed that most strains are resistant to penicillin (93.8%), tetracycline (68.7%), and kanamycin (53.2%). In addition, 37.5% (12 out of 32) of isolates were methicillin-resistant *S. epidermidis* (MRSE). All strains were susceptible to minocyclin, pristinamycin, nitrofurantoin, and vancomycin. Twenty-three strains were found to be positive for the intercellular adhesion gene (*ica*). Of these 23, 19 strains were *mecA* positive. In contrast, 4 *icaA/icaD* positive strains were found to be *mecA* negative. The statistical analyses show no significant difference between the presence of *mecA* gene and *icaA/icaD* loci (*P* = 0.327). The presence and expression of *ica* genes can clarify the adhesion mechanisms in the pathogenesis of infections associated with medical devices, and assist in the development of new preventive and therapeutic measures to eradicate biofilm in hospitals. Bacterial adhesion with biomaterials has been suggested to play a crucial role in the induction of severe nosocomial infections in hospitals (15). In a recent study, it has been shown that a high frequency of *S. epidermidis* (72%) isolated from biomaterial carries the *icaA/icaD* gene (16). Three *ermA* positive strains (S21, S33, and S48) and 8 *ermB* positive strains under study were *icaA/icaD* positive. Furthermore, we found that 14 of the 17 *ermC* positive strains were *icaA/icaD* positive, and 15 of the 22 *msrA* positive strains were also *icaA/icaD* positive. In addition we found that 96% (22 out of 23) were resistant to at least two antibiotics. Three *icaA/icaD* positive strains were resistant to 10, 12, and 13 antibiotics (S15, E15, and S26 respectively). Most reports demonstrate that the adherent strains are multi-resistant to antibiotics (17). Recent studies have demonstrated that the biofilm-producing *icaADBC* operon is a typical marker of *S. epidermidis* strains obtained from device-related infections (18). Moreover, it has previously been found in animal models that experimental infections by biofilm-producing *S. epidermidis* strains are more serious than those induced by biofilm-negative strains (19). Control of the spread of drug-resistant genes in staphylococci is important for limiting its dissemination in hospitals (20,21). Previous studies have shown that adherent *S. epidermidis* strains are responsible for bacteremia and implant-associated orthopedic infections (22,23). Methicillin-resistant *Staphylococcus* displays resistance to a variety of antimicrobial agents, including non β-lactam antimicrobials, and leads to a high mortality rate in immunocompromised hosts. Investigating the spread of drug resistance genes in staphylococci is important to the control of its dissemination (21).

We have compared oxacillin and erythromycin susceptibilities among strains and found that 8 of 12 oxacillin-resistant strains were erythromycin-resistant and contain at least 1 erythromycin resistance gene.

**Table 2. Correlation between oxacillin resistance and the presence of the resistance gene *mecA* in *S. epidermidis***

<table>
<thead>
<tr>
<th>No. of strains</th>
<th>PCR detection for <em>mecA</em> gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxacillin-susceptible</td>
<td><em>mecA</em>-positive</td>
</tr>
<tr>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>oxacillin-resistant</td>
<td>12</td>
</tr>
</tbody>
</table>
The antibiotic susceptibility data obtained with the ATB Staph system with those obtained by PCR assays. The antibiotic susceptibility of the 32 strains showed multi-resistance against the 18 previously cited antibiotics. Oxacillin resistance was observed in 12 of the 32 (37.5%) strains when tested by ATB Staph. Approximately 78.1% of the isolated S. epidermidis strains have the mecA gene. Phenotypically oxacillin-sensitive and mecA-positive isolates lead us to believe that the gene is present but the phenotype is not. In this study we found 16 strains susceptible to oxacillin and carrying the mecA gene. This result was contradictory to that of Raimundo et al. (24), which showed complete agreement between the presence or absence of the mecA gene and the interpretation of oxacillin disc susceptibility test. Guisti et al. suggest that the mecA-negative strains currently express phenotypic oxacillin resistance mediated by a mechanism other than the presence of the mecA gene (25).

The mecA expression was influenced by various factors. Conventional techniques may be inadequate in the investigation of resistance to antibiotics in staphylococci (13). Zambardi et al. have suggested that the sensitivity of oxacillin resistance detection depends upon the salt concentration in the medium with an optimum concentration (2%) (26). In addition, they have shown that the ATB test is the most effective method (sensitivity, 89.8%), and the search for the mecA gene by PCR represents a very interesting method that detects 96.9% of oxacillin-resistant CoNS strains (26,27). In addition, three genes resistant to oxacillin and lacking the mecA gene were found, suggesting an alternative mechanism to drug resistance.

Erythromycin resistance in staphylococci is predominantly mediated by erythromycin resistance methylase encoded by erm genes (6). On the other hand, the prevalence of ermA, ermB, and ermC genes in Tunisian S. epidermidis strains was investigated, and it was found that only 6 strains (21.87%) are susceptible to erythromycin but do not contain any erythromycin resistance genes. In 5 strains (E7, E20, S23, S27, and S40), the ermA and ermC gene were not detected, although the strains were resistant. The erythromycin resistance might be due to the presence of msrA or ermB gene, as previously described in CoNS (7). In addition, the strain E7 was resistant to erythromycin but does not carry any erythromycin resistance gene, according to Table 3. This result might be due to the location of these genes in small plasmids, which are occasionally lost. Sekiguchi et al. (21) have also found discordance between the presence of erythromycin genes and phenotypic sensitivity. It has been shown that conventional techniques may be inadequate in the investigation of resistance to antibiotics in staphylococci (13,28). The ermC gene that is responsible for constitutive or inducible resistance to erythromycin is generally located on small plasmids (29). Several studies have been performed using PCR and multiplex PCR techniques that identify resistance genes to various antibiotics in staphylococci (14,30). Lim et al.
(14) detected the 

**ermA** gene primarily in *S. aureus* isolates (82.5%) and **ermC** mainly in CoNS (47.2%). In addition, 4 *S. epidermidis* strains were **ermA** positive (12.5%). A similar incidence has been reported in CoNS isolated from various sites and specimens. On the other hand, our result was contradictory to that of Thakker-Varia et al., who reported a higher incidence for **ermA** in CoNS (31). In addition, 10 strains carried the **ermB** gene, whereas 53.1% of *S. epidermidis* strains were found to carry **ermC**. Martineau et al. have also reported a high incidence of CoNS strains carrying the **ermC** gene (13). Furthermore, the **msrA** gene was present in 22 of 32 (68.75%) strains of *S. epidermidis*. In contrast the **mef** gene was absent in all tested strains. These results are similar to those of a recent study, which demonstrates the presence of a high level of **ermA** and **ermC** gene in both methicillin-resistant *Staphylococcus aureus* (MRSA) and MRCoNS (26). A recent study suggests that antimicrobial drug treatment affects our indigenous microbiota and can give rise to long-term colonization with resistant populations (32).

In clinical settings, the identification of bacteria using classical methods and determination of its susceptibility to antibiotics generally require 48 h, whereas the use of PCR assay for the detection of antibiotic resistance genes is more rapid than the other traditional methods. The molecular epidemiological tools are helpful for understanding the transmission patterns for the control of nosocomial infection assessed by CoNS. It can help the clinician to determine the species involved in suspected staphylococcal sepsis and also their susceptibility to different antibiotics.

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