Molecular typing and characterization of macrolide, lincosamide and streptogramin resistance in \textit{Staphylococcus epidermidis} strains isolated in a Mexican hospital

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Staphylococcus epidermidis is a normal commensal of skin that has become a serious clinical problem because of the combination of increased use of intravascular devices and an increasing number of hospitalized immunocompromised patients. In addition, there is a lack of information pertaining to resistance to macrolide, lincosamide and streptogramin type B (MLS\textsubscript{B}) in developing countries, including Mexico. The aim of this study was to investigate the incidence of resistance to MLS\textsubscript{B} antibiotics in isolates of \textit{S. epidermidis} obtained in the General Hospital of Acapulco in Mexico. Susceptibility to erythromycin, clindamycin and quinupristin–dalfopristin was tested by a diffusion test, and MICs to oxacillin, erythromycin and lincomycin were determined. Differentiation between MLS\textsubscript{B} phenotypes was performed by a double disc diffusion test. A total of 38 of the 47 strains of \textit{S. epidermidis} isolated from nosocomial infections were resistant to oxacillin [meticillin-resistant \textit{S. epidermidis} (MRSE)]. The phenotypes obtained were: 18 constitutive MLS\textsubscript{B}, 3 inducible MLS\textsubscript{B}, 6 macrolide streptogramin and 4 lincosamide; 7 strains were susceptible to MLS\textsubscript{B} antibiotics. The genes associated with resistance were detected by PCR. Genotyping showed a predominance of the \textit{ermA} gene followed by genes \textit{ermC} and \textit{msrA}. The frequency of the genes detected varied slightly from results that have been reported in isolates from other countries. Clonal types were identified by PFGE and revealed the dissemination of two major clones of MRSE in the Mexican hospital. This is believed to be the first report in Mexico on the genes associated with the MLS\textsubscript{B} resistance phenotype in \textit{S. epidermidis}, in addition to observing a wide distribution of clonal types in the General Hospital of Acapulco, Mexico.

INTRODUCTION

\textit{Staphylococcus epidermidis} has emerged as a major pathogen in nosocomial infections. The increasing use of indwelling devices along with changes in patient populations, hospital environments, medical practice and antibiotic policies have turned these micro-organisms into the leading cause of hospital-acquired bacteraemia (Burnie et al., 1997; Rosenthal et al., 2008; Schoenfelder et al., 2010).

The members of the class of antibiotics comprising macrolides, lincosamides and streptogramin type B (MLS\textsubscript{B}) are chemically different, but have similar inhibitory effects on bacterial protein synthesis (Leclercq & Courvalin, 1991). These antibiotics are widely used in the treatment of Gram-positive infections, especially meticillin-resistant staphylococci, and are the best therapeutic alternative for patients who are hypersensitive to penicillin. However, the widespread use of these antibiotics has caused increased resistance among staphylococci (Hamilton-Miller & Shah, et al., 1997; Rosenthal et al., 2008; Schoenfelder et al., 2010).
Resistance to erythromycin is usually associated with cross-resistance to other MLSB. The most common resistance mechanisms in staphylococci are target site modification and active drug efflux. Target site modification is mediated by the ermA, ermB and/or ermC genes (Gatermann et al., 2007). In addition, the phenotypic expression of MLSB resistance can be inducible or constitutive (Jorgensen et al., 2004). In contrast, active efflux of macrolides is affected by membrane proteins encoded by the msrA gene, which is specific for 14- and 15-member macrolides and streptogramin type B (MS phenotype); lincosamides and streptogramin A remain unaffected. The lnuA gene confers resistance only to lincosamides, whilst the vga gene has been characterized as a determinant of streptogramin A resistance (Lina et al., 1999; Roberts et al., 1999).

The use of accurate and discriminatory typing methods is critical for epidemiological research and to define the origin of infections. PFGE of Smal chromosomal DNA fragments is a sensitive method available for the discrimination of staphylococci (Tenover et al., 1995). PFGE findings might be useful in staphylococci to estimate local dissemination in an institution, which in turn can be used to improve the accuracy of the clinical diagnosis of bacteraemia (Senger et al., 2007). To determine the status of MLSB resistance and provide a guideline for antibiotic usage, we investigated the frequency of MLSB resistance in S. epidermidis strains collected from a Mexican hospital.

**METHODS**

**Bacterial isolates.** A total of 47 consecutive clinical isolates of S. epidermidis were obtained from patients with a nosocomial infection in the General Hospital of Acapulco, Mexico, between January 2002 and December 2004. One bacterial isolate per patient was included. During the study period, 36% of nosocomial infections were associated with 182 isolates of Staphylococcus spp., of which 47 strains were identified as S. epidermidis. Isolates were identified by standard procedures: Gram staining, growth on manitol salt agar (BBL; Becton Dickinson), catalase and coagulase tests, and complemented with the API Staph system (bioMérieux). All isolates were stored at −70 °C in brain–heart infusion broth with 30% glycerol until further analysis.

**Antimicrobial susceptibility testing.** Susceptibility to the antibiotics erythromycin, clindamycin, clarithromycin, azithromycin and quinupristin–dalfopristin was performed by a disc diffusion susceptibility test (CLSI, 2009a). MICs to oxacillin, clindamycin and erythromycin (Sigma) were determined by dilution antimicrobial susceptibility tests for bacteria that grow aerobically (CLSI, 2009b). The results were interpreted according to the Clinical and Laboratory Standards Institute recommendations (CLSI, 2009c). To differentiate MS and MLSB phenotypes for all isolates, a disc approximation test was performed for detection of inducible clindamycin resistance in Staphylococcus spp. (Yilmaz et al., 2007) by placing a 15 μg erythromycin disc (Oxoid) 15 mm away from the edge of a 2 μg clindamycin disc (Oxoid) on Mueller–Hinton agar plates (Oxoid) and incubating for 24 h at 35 °C. Following incubation, organisms that showed flattening of the clindamycin zone (called a ‘D’ zone) were considered to have inducible clindamycin resistance [inducible MLSB (iMLSb)], whereas isolates that did not show flattening of the clindamycin zone were reported as clindamycin sensitive. S. epidermidis isolates resistant to both erythromycin and clindamycin were defined as having constitutive MLSB resistance (cMLSb). Staphylococcus aureus ATCC 25923 was used as a negative control.

**PCRs.** Chromosomal DNA from all isolates was extracted as described by Vannuffel et al. (1995). The DNA was used as a template for amplification of seven resistance genes: meca, ermA, ermB, ermC, msrA, lnuA and vga. The primer pairs and amplification conditions used for PCR are listed in Table 1. Each 50 μl reaction mixture contained 0.25 mM each dNTP, 50 pmol primers, 10 mM Tris/HCl (pH 8.8), 1.5 mM MgCl2, 50 mM KCl, 0.1 % Triton X-100, 1.25 U DNA polymerase (Applied Biosystems) and 10 μl DNA sample. Amplification was carried out in a GeneAmp PCR System 2400 thermocycler (Applied Biosystems) with an initial 5 min denaturation at 94 °C and 10 min final extension at 72 °C. DNA was detected by gel electrophoresis on 1.5 % agarose gels run for 60 min at 95 V and stained with ethidium bromide (1 μg ml⁻¹). S.

**Table 1. Primer sequences and amplification conditions used in this study**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’→3’)</th>
<th>Product (bp)</th>
<th>Cycles (and conditions)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ermA</td>
<td>GTTCAAGAACAAATCAATACAGAG</td>
<td>421</td>
<td>30 (30 s, 94 °C; 30 s, 52 °C; 1 min 72 °C)</td>
<td>Lina et al. (1999)</td>
</tr>
<tr>
<td>ermB</td>
<td>CCGTTTACGAAATGGAACAGTTAAGGCC</td>
<td>359</td>
<td>30 (30 s, 94 °C; 30 s, 55 °C; 1 min 72 °C)</td>
<td>Lina et al. (1999)</td>
</tr>
<tr>
<td>ermC</td>
<td>GCTATTATTGTTTAAATCCTCAATTTCC</td>
<td>572</td>
<td>30 (30 s, 94 °C; 30 s, 52 °C; 1 min 72 °C)</td>
<td>Lina et al. (1999)</td>
</tr>
<tr>
<td>msrA</td>
<td>GCCACAATAAAGATGGTTAAGGCC</td>
<td>940</td>
<td>25 (1 min, 94 °C; 1 min, 50 °C; 90 s, 72 °C)</td>
<td>Lina et al. (1999)</td>
</tr>
<tr>
<td>lnuA</td>
<td>GTTTGGGCTTGGGTTAGATGGTAAAACTGG</td>
<td>323</td>
<td>30 (30 s, 94 °C; 30 s, 57 °C; 1 min 72 °C)</td>
<td>Lina et al. (1999)</td>
</tr>
<tr>
<td>vga</td>
<td>CCAGAACCTTGATTTAGCAAGATGAA</td>
<td>470</td>
<td>30 (30 s, 94 °C; 30 s, 54 °C; 1 min 72 °C)</td>
<td>Lina et al. (1999)</td>
</tr>
<tr>
<td>mecA</td>
<td>TGCGTATCGTGTCACACTCG</td>
<td>310</td>
<td>30 (1 min, 94 °C; 1 min, 56 °C; 3 min 72 °C)</td>
<td>Vannuffel et al. (1995)</td>
</tr>
</tbody>
</table>
**RESULTS**

**Antimicrobial susceptibility and the mecA gene**

A total of 38 out of 47 *S. epidermidis* isolates (80.8%) from nosocomial infections were resistant to oxacillin [meticillin-resistant *S. epidermidis* (MRSE)] and also were positive for the mecA gene (Table 2). Of the 9 isolates susceptible to oxacillin, only 2 had a macrolide and lincosamide phenotype; thus, only the 38 resistant isolates were included for further characterization. The antibiotic profiles for resistance to erythromycin, clindamycin and quinupristin–dalfopristin are shown in Table 2. Eighteen (47%)
presented the cMLSB phenotype, three (8%) the iMLSB phenotype, six (16%) the MS phenotype, four (11%) the L phenotype and seven (18%) were susceptible to all antibiotics. The MIC50 value to erythromycin was high (64 μg ml⁻¹) and the MIC50 level to clindamycin was low (1 μg ml⁻¹). However, the MIC90 level of both was high (>256 μg ml⁻¹).

**MLSβ resistance genotypes**

A total of 33 out of 38 MRSE had the genes ermA, ermB, ermC, msrA, lnuA and/or vga, which have been associated with resistance to MLSβ antibiotics. The major determinant in MRSE was ermA, which was detected in 12 (32%) isolates and was frequently associated with vga, but it was not present in iMLSB isolates. The gene ermC was observed in ten strains, whereas ermB was located in a single strain of *S. epidermidis*. Finally, five clinical isolates were negative for PCR, and three susceptible strains had the resistance genes ermA + lnuA, lnuA and msrA, respectively (Table 2).

**Clonal distribution of MRSE**

From the 38 isolates, we obtained two major patterns by PFGE analysis: pattern A (including ten subtypes, A1–A10), and pattern B (with seven subtypes, B1–B7) (Fig. 1). Pattern A included 15 isolates and pattern B 9 isolates, whilst 14 isolates were not related (Table 2). In addition, the dendrogram showed similar rates of 58–100%, where the two major clusters appeared to be associated with identity percentages of >70% (Fig. 2). Although there was no relationship between phenotype and genotype assays, pulsotype A predominantly expressed the MLSβ susceptible and the MS resistance phenotypes. In contrast, pulsotype B mainly expressed the cMLSB phenotype. Three isolates had the ermC gene and expressed the iMLSB phenotype but had no epidemiological relationship. A total of 13 of the 17 MRSE isolates with the cMLSB phenotype carried at least one member of the *erm* gene family. Independent of the phenotype, the *SmaI* genomic fingerprints of the MRSE isolates from the General Hospital of Acapulco were distributed among different wards, mainly in the paediatric ward, and were unrelated from 2002 to 2004 (Table 2).

**DISCUSSION**

*S. epidermidis* is a leading cause of infectious disease in hospital settings, and when found in the blood has been associated with central venous catheters (Burnie et al., 1997; Brito et al., 2009). Multiresistant strains to different classes of antibiotic have been reported to acquire resistance to macrolides and related antibiotics. Therefore, the treatment of infections due to MRSE and their eradication are very difficult. Constant monitoring of these strains is essential to control their spread (Miragaia et al., 2002). The displayed frequency between the phenotypes of resistance to macrolides, lincosamides and streptogramins varies among different hospitals and geographical areas. For coagulase-negative staphylococci (CNS) in London (Hamilton-Miller & Shah, 2000), a resistance of 55% [macrolide (M)], 12% [lincosamide (L)] and 0% [streptogramin type B (S)] has been reported, whilst for other European countries (Schmitz et al., 1999) results were 75% (M), 54% (L) and 0% (S). The results obtained in this study showed a resistance of 64% (M), 42% (L) and 32% (S). Thus, the resistance range obtained for macrolides and lincosamides was similar to that reported, but was higher for the streptogramins, which was surprising as these antibiotics are not used in Mexico. The iMLSB phenotype was determined in only three isolates (7.8%), representing a low frequency compared with 21.9% (Yilmaz et al., 2007) and 35% (Schreckenberger et al., 2004) reported previously.

The CNS isolates were analysed for the presence of *erm* methylase and the *msrA* efflux pump by PCR. The *ermA* gene was the resistance determinant detected most frequently in *S. epidermidis* isolates, and similar results have been reported by other authors (Ardic et al., 2005).

![Fig. 1. Representative gel from SmaI macro-restriction fragments of *S. epidermidis* clinical clones identified in this work. This analysis was performed on two gels; one is shown here containing 27 samples, representing the subtypes obtained. Lanes 1 and 29, molecular size standard (λ oligomers). Letters at the bottom indicate the PFGE pattern. NR, Not related.](http://jmm.sgmjournals.org)
However, this result is in contrast to other findings, where the \textit{ermC} gene was the main resistance determinant observed in CNS, including \textit{S. epidermidis} (Lim \textit{et al.}, 2002; Cetin \textit{et al.}, 2010). In this study, only one isolate had the \textit{ermB} gene, which has been detected by other groups in a low percentage of staphylococci (Hamilton-Miller \& Shah, 2000; Chaieb \textit{et al.}, 2007). In contrast to Novotna \textit{et al.} (2005) who confirmed a high occurrence (26 \%) of the \textit{msrA} gene for \textit{S. epidermidis}, we found a frequency of 12.5 \%. In addition, the methylase genes \textit{ermA}, \textit{ermB} and \textit{ermC}, as well as the \textit{msrA} gene, were detected alone or in different combinations in this study and in another (Lüthje \& Schwarz, 2007). Two of the susceptible strains had the \textit{lnuA} gene, which was similar to the study of Lüthje \& Schwarz (2006) who observed the presence of this gene in \textit{S. epidermidis} isolates susceptible to erythromycin; however, they showed that the presence of the \textit{lnuA} gene was associated with a specific resistance to pirlimycin, a lincosamide of veterinary use, which was not used in this study. Two strains with the genes \textit{ermA} and \textit{msrA} showed a susceptible phenotype, which may be due to a defect in the expression of the resistance mechanisms involved. Finding such evidence in the literature is complicated because studies that address the detection of genes are exclusively for strains that are resistant to MLSB antibiotics.

The persistence and dissemination of \textit{S. epidermidis} clones within hospitals have been documented as having their origin in the community or being endemic in the hospital (Widerström \textit{et al.}, 2006; Brito \textit{et al.}, 2009). Also, it has been suggested that antibiotic use is a factor in the selection of CNS clones (Krediet \textit{et al.}, 2004). The results presented here demonstrated that isolates corresponded to clone B with a cMLS\textsubscript{B} phenotype as well the \textit{ermA} gene, whereas strains corresponding to clone A presented the MS and susceptibility phenotypes. To our knowledge, this is the first study oriented towards characterizing \textit{S. epidermidis} strains collected in a Mexican hospital. In conclusion, the isolates used in this study showed a relatively high rate of resistance to MLS\textsubscript{B} antibiotics, presenting a constitutive behaviour. The \textit{ermA} gene was the most frequently detected among all the isolates. Infections by \textit{S. epidermidis} isolates from the General Hospital of Acapulco, Mexico, formed two clusters of CNS molecular types that were able to persist in different nosocomial areas at least for 3 years.

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