High Frequency of Macrolide Resistance Mechanisms in Clinical Isolates of *Corynebacterium* Species

Alberto Ortiz-Pérez, Nieves Z. Martín-de-Hijas, Jaime Esteban, María Isabel Fernández-Natal, José Ignacio García-Cia, and Ricardo Fernández-Roblas

The genus *Corynebacterium* includes a high number of species that are usually isolated from human skin as saprophytes. However, these microorganisms have also been reported as infectious agents in a broad group of patients and have showed broad-spectrum resistance. We studied the susceptibility profiles against macrolides, clindamycin, and streptogramins of 254 clinical strains belonging to the species *Corynebacterium urealyticum* (120), *Corynebacterium amycolatum* (66), *Corynebacterium jeikeium* (17), *Corynebacterium striatum* (20), *Corynebacterium coyleae* (12), *Corynebacterium aurimucosum* (11), and *Corynebacterium afermentans* subsp. *afermentans* (8). The MLS\(_B\) phenotype was detected in 186 strains and was associated with the presence of methylase enzymes codified by the *erm*(X) gene in 171 strains. The *erm*(B) gene was only detected in two *C. urealyticum* strains. Fourteen strains showed macrolide resistance, but they did not carry *erm* genes. *mef* genes were not detected despite eight *C. amycolatum* strains showed the M phenotype. Also, the presence of hydrolytic enzymes codified by *ere*(B) was evaluated, but all results were negative. Resistance to macrolide in *Corynebacterium* sp. is mainly due to the presence of *erm*(X) methylase, although other resistance mechanisms could be involved.

**Introduction**

*Corynebacterium* species are ubiquitous organisms that belong to the human skin microbiota. They are frequently isolated from clinical samples, although their presence is usually considered as a colonization or contamination of the sample. However, several members of this genus are cause of infections, some of them associated with the use of biomaterials. The species *Corynebacterium urealyticum*, *Corynebacterium amycolatum*, *Corynebacterium jeikeium*, and *Corynebacterium striatum* have been reported as the most important pathogens of this genus. In recent years, other species have also been recognized as human pathogens. The increasing clinical importance of these microorganisms makes necessary the knowledge of their antibiotic susceptibility. Different studies have showed high resistance rates against several antimicrobials, such as β-lactams, aminoglycosides, quinolones, macrolides, lincosamides, and tetracyclines. The studied strains showed only uniform susceptibility to glycopeptides and linezolid. The high level of macrolide resistance is a matter of particular concern. Macrolides are frequently used in infections that can be caused by these microorganisms, and are an alternative to β-lactams and tetracyclines. Macrolide resistance is mainly due to methylase enzymes that are codified by *erm* genes. Other resistance mechanisms such as mutations in 23S rRNA domain V, modifications of proteins L4 and L22, efflux pump systems, and hydrolytic enzymes have been reported in different genera. Despite all these data, only a few reports have studied this topic in *Corynebacterium* sp. The objective of this study was to evaluate the mechanisms implicated in macrolide resistance among clinical isolates of *Corynebacterium* species.

**Materials and Methods**

**Bacterial strains**

Bacterial strains were isolated from clinical samples of patients with infection and from a skin colonization study in hospitalized patients presented at the Hospital Fundación Jiménez Díaz de Madrid, Spain, and the Complejo Asistencial de León, Spain, between January 1985 and December 2005. In this study, one isolate per patient was used. The strains were identified by the API Coryne System (bioMérieux, Marcy l’Etoile, France) using the instructions provided.
by the manufacturer. All the strains belong to the species *C. amycolatum*, *C. striatum*, *Corynebacterium coyleae*, *Corynebacterium aurimucosum*, and *Corynebacterium afermentans* subsp. *aferrmentans*, as well as a randomly selected strains from the species *C. urealyticum* and *C. jeikeium*, and were also identified by 16S rDNA sequence analysis using the protocol described by Fernández-Natal et al. Strains were kept frozen in skim milk at −20°C.

**Antimicrobial susceptibility**

*In vitro* susceptibility testing against erythromycin, clarithromycin, azithromycin, and spiramycin, and quinupristin/dalfopristin was conducted using E-test strips (AB, Biodisk, Solna, Sweden) using the previously described protocol. The susceptibility of spiramycin was studied using commercial disks of 100 μg (bioMérieux). All the studies were performed on Mueller–Hinton agar supplemented with 5% sheep blood in a normal atmosphere at 37°C during 24–48 hours.

Susceptibility to erythromycin, clarithromycin, and quinupristin/dalfopristin was assessed using the breakpoints described for *Corynebacterium* sp. by the Clinical and Laboratory Standards Institute (CLSI). To analyze the susceptibility of clarithromycin, azithromycin, and spiramycin, breakpoints reported for *Staphylococcus* sp. were used. *Staphylococcus aureus* ATCC 29213 and ATCC 25923 collection strains were used for quality control purposes.

**Detection of the resistance genes**

The strains were grown on blood agar plates during 24–48 hours at 37°C in normal atmosphere and checked for purity. One loop was resuspended in 200 μl of sterile distilled water. Suspension was boiled during 20 minutes at 100°C and subsequently centrifuged at 12,000 rpm for 15 minutes. Supernatant (150 μl) was used for PCR studies.

PCR was performed using commercial puRe Taq Ready-To-Go PCR Beads® (Amersham Biosciences, Buckinghamshire, United Kingdom). The primers used for detection of the *erm* gene were Cerm 1 (5′-GAC ACG GCC GTC ACG AGC AT-3′) and Cerm 2 (5′-GCG GCC GAG CGA CTT CC-3′); for the *erm(A)* gene, Erm A-1 (5′-TCT AAA AAG CAT GTA AAA GAA-3′) and Erm A-2 (5′-CTT CGA TAG TTT ATT AAT AGT-3′); for the *erm(B)* gene, Erm B-1 (5′-GAA AAG GTA CTC AAC CAA ATA-3′) and Erm B-2 (5′-AGT AAC GGT ACT TAA ATT GTT TAG TCC-3′); and for the *mef(A-*E) gene, Mef A-1 (5′-AGT ATC ATT ATT CAC TAG TGC-3′) and Mef A-2 (5′-TTT TTC TGG TAC AAG TGG-3′). The *erm(X)* gene amplification was performed according to the protocol described by Rosato et al. For all other genes we applied PCR conditions previously reported for *Streptococcus* sp. The amplification products were detected by electrophoresis in 3% agarose. The size of the amplicons was estimated using Biogen software (Vilber-Lourmat, Marne-la-Vallée, France) compared with a commercial molecular weight ladder (Bio Rad Laboratories, Hercules, CA). The *erm(X)* and *erm(B)* amplicons were subsequently purified and sequenced with the primers Cerm 1, Cerm 2, Erm B-1, and Erm B-2. Nucleotide sequences were analyzed with BLAST software and compared with previously reported sequences (GenBank, NCBI).

**Erythromycin enzymatic hydrolysis**

To determine the presence of the *ere(B)* gene and its hydrolytic effect over erythromycin, we have used the protocol described by Wondrack et al. The growth of the erythromycin-sensitive *S. aureus* ATCC 25923 strain was evaluated in blood-agar plates after 24 hours at 37°C in a normal atmosphere with erythromycin disks of 40 and 400 mg/L. Previously, the disks and a suspension of different *Corynebacterium* strains were incubated together to evaluate the hydrolysis of this antibiotic. The *Escherichia coli* strain was used as a positive control. This strain has a plasmid that inactivates the erythromycin and was provided by Dr. Courvalin (Pasteur Institute, Paris, France). Distilled water was used as a negative control.

**Statistical analysis**

Erythromycin susceptibility and resistance rates among *Corynebacterium* species were analyzed by means of exact Fisher test. All statistical calculations were performed using SPSS 10.0 Software (SPSS, Inc., Chicago, IL).

**Results**

Two hundred fifty-four *Corynebacterium* spp. strains were included in this study: 120 *C. urealyticum*, 66 *C. amycolatum*, 20 *C. striatum*, 17 *C. jeikeium*, 12 *C. coyleae*, 11 *C. aurimucosum*, and 8 *C. afermentans* subsp. *aferrmentans*. The results of the *in vitro* susceptibility study are shown in Table 1. Among the macrolide-resistant strains, 186 showed an MLSB phenotype, with a high level of resistance against macrolides and clindamycin, while 8 strains showed the M phenotype, with low level resistance against 14- and 15-membered macrolides but with a high level of resistance against macrolides and clindamycin. Six strains showed the M phenotype and susceptibility to 16-membered macrolide, clindamycin, and quinupristin-dalfopristin.

The amplification product of the *erm(X)* gene was detected in 175 strains: 171 showed MLSB resistance, 2 strains (one *C. striatum* and one *C. aurimucosum* strains) were susceptible against all studied antibiotics, and 2 *C. amycolatum* strains were resistant to erythromycin, clarithromycin, and azithromycin but susceptible against spiramycin, clindamycin, and quinupristin-dalfopristin (M phenotype). No amplification products were detected in 14 strains, although they showed resistance to macrolide and clindamycin. There were also six *C. amycolatum* strains showing the M phenotype and without any of the *erm* amplicons studied.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Susceptibility, number of strains (%)</th>
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<tbody>
<tr>
<td></td>
<td>S</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>60 (23.62)</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>61 (24.01)</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>60 (23.62)</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>62 (26.77)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>60 (23.62)</td>
</tr>
<tr>
<td>Quinupristin/ dalfopristin</td>
<td>229 (90.20)</td>
</tr>
</tbody>
</table>

S, susceptible; I, intermediate; R, resistant.

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**Table 1. Susceptibility of 254 Corynebacterium Strains Studied to MLS Antibiotics**

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product of \(\text{erm}(B)\) gene was detected in two \(\text{C. urealyticum}\) strains, and in one of them both \(\text{erm}(X)\) and \(\text{erm}(B)\) amplicons were detected. The \(\text{erm}(A)\) and \(\text{mef}(A-E)\) amplification products were not detected in any of the studied \(\text{Corynebacterium}\) strains (Table 2).

The \(\text{C. striatum}\) strains were more susceptible against erythromycin compared with all other species, although this difference was only statistically significant against \(\text{C. amycolatum}\) strains \((p = 0.011, \text{Fisher's exact test})\), but not against \(\text{C. urealyticum}\) strains \((p = 0.103)\) or \(\text{C. jeikeium}\) strains \((p = 0.3)\). The number of \(\text{C. coyleae, C. aurimucosum, and C. afermentans}\) strains was too low for a proper statistical analysis.

The \(\text{erm}(X)\) amplicon sequence was over 390 pair of bases and showed a nucleotide identity between 90% and 99% with the previously described sequence (Fig. 1). The \(\text{erm}(B)\) amplicon sequence of \(\text{C. urealyticum}\) was 639 pair of bases, showing a nucleotide identity of 99% with respect to other

<table>
<thead>
<tr>
<th>Species</th>
<th>Number</th>
<th>(\text{erm}(X))</th>
<th>(\text{erm}(B))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{C. urealyticum})</td>
<td>120</td>
<td>82(^a)</td>
<td>2(^a)</td>
</tr>
<tr>
<td>(\text{C. amycolatum})</td>
<td>66</td>
<td>49(^b)</td>
<td>0</td>
</tr>
<tr>
<td>(\text{C. striatum})</td>
<td>20</td>
<td>11(^c)</td>
<td>0</td>
</tr>
<tr>
<td>(\text{C. jeikeium})</td>
<td>17</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>(\text{C. coyleae})</td>
<td>12</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>(\text{C. aurimucosum})</td>
<td>11</td>
<td>8(^c)</td>
<td>0</td>
</tr>
<tr>
<td>(\text{C. afermentans})</td>
<td>8</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)One strain carried the genes \(\text{erm}(X)\) and \(\text{erm}(B)\) simultaneously. 
\(^b\)Two strains showed the M phenotype despite carrying the \(\text{erm}(X)\).

\(^c\)One strain was susceptible despite being \(\text{erm}(X)\) gene positive.

FIG. 1. Analysis of \(\text{erm}(X)\) determinant in \(\text{Corynebacterium urealyticum}\). PCR products of amplification using primers Cerm 1 and Cerm 2. Lane 1, strain A-6; lane 2, strain A-7; lane 3, strain A-8; lane 4, strain A-9; lane 5, strain A-10; lane 6, strain A-11; lane 7, strain A-12; lane 8, strain A-13; lane 9, strain A-14; lane 10, DNA ladder; lane 11, strain A-15; lane 12, strain A-16; lane 13, strain A-17; lane 14, strain A-18; lane 15, strain A-20; lane 16, strain A-21; lane 17, strain A-22; lane 18, strain A-23; lane 19, distilled water (negative control); lane 20, \(\text{Corynebacterium jeikeium}\) CB-396 (positive control). pb, pair of bases.

FIG. 2. Analysis of \(\text{erm}(B)\) determinant. PCR products of amplification using primers Erm B-1 and Erm B-2. Lane 3, \(\text{Corynebacterium urealyticum}\) CB-257; lane 11, DNA ladder; lanes 16 and 17, \(\text{Streptococcus agalactiae}\) 637 (positive control); lane 18, distilled water (negative control); Other lanes, negative strains. pb, pair of bases.
would potentially lead to a constitutive expression of the methylase. In an analogous way, deletions or mutations in the codified region could make the \textit{erm}(X) an inoperative gene.

Fourteen isolates showed an MLS\textsubscript{B} resistance phenotype despite being negative for all \textit{erm} genes. This must be due to the presence of other resistance mechanisms. In this sense, mutations in 23S rRNA domain V and alterations of proteins L4 and L22 have originated an MLS\textsubscript{B} resistance phenotype in \textit{Streptococcus pneumoniae}.\cite{6,30} The presence of these mutations in \textit{Corynebacterium} sp. could be an explanation for the resistance to macrolides, although more studies would be necessary to evaluate this possibility.

The amplification products of the \textit{erm}(B) gene were detected in two \textit{C. urealyticum} strains. The presence of this gene has already been described in group A \textit{Corynebacterium} strains.\cite{17} The prevalence of this gene was very low and highly contrasts with the high percentage of \textit{erm}(X)-gene-positive strains. Besides, there was a strain that carried the \textit{erm}(X) and \textit{erm}(B) genes simultaneously, a characteristic previously reported in \textit{Staphylococcus}, where the presence of several \textit{erm} genes is very common.\cite{1}

Eight \textit{C. amycolatum} strains showed the M phenotype. This phenotype is usually mediated by efflux systems that are usually present in \textit{Streptococcus} and are codified mainly by the \textit{mef} genes.\cite{6} However, in our study, no amplification products of the \textit{mef} gene were detected, although this gene has been previously reported in the genus \textit{Corynebacterium}.\cite{17} Other efflux pump genes such as \textit{msr} have also been recently reported in this genus,\cite{19} but they were not studied in our report.

The effect of the \textit{ere}(B) gene, related with hydrolysis of antibiotics, was studied in some strains. The presence of this gene is probably unusual among \textit{Corynebacterium} sp., because it has been mainly described for Gram-negative bacteria.\cite{22}

Conclusions

According to our data \textit{erm}(X) is the most important gene implicated in the macrolide resistance of all \textit{Corynebacterium} species analyzed in our study. Other resistance genes like \textit{erm}(B) must also be implicated in this resistance, although its presence seems to be unusual.

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Disclosure Statement

No conflicts of interest exist for any authors.

References


4. [CLSI] Clinical and Laboratory Standards Institute. 2006. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria; approved guideline. CLSI, Wayne, PA.


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