Phenotypes and genotypes of erythromycin-resistant \textit{Streptococcus pyogenes} strains isolated from invasive and non-invasive infections from Mexico and the USA during 1999–2010\textsuperscript{2,\#}

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Objective: To compare the prevalence, phenotypes, and genes responsible for erythromycin resistance among \textit{Streptococcus pyogenes} isolates from Mexico and the USA.

Methods: Eighty-nine invasive and 378 non-invasive isolates from Mexico, plus 148 invasive, 21 non-invasive, and five unclassified isolates from the USA were studied. Susceptibilities to penicillin, erythromycin, clindamycin, ceftriaxone, and vancomycin were evaluated according to Clinical and Laboratory Standards Institute (CLSI) standards. Phenotypes of erythromycin resistance were identified by triple disk test, and screening for mefA, ermA, and ermA genes was carried out by PCR.

Results: All isolates were susceptible to penicillin, ceftriaxone, and vancomycin. Erythromycin resistance was found in 4.8% of Mexican strains and 5.2% of USA strains. Phenotypes in Mexican strains were 95% M and 5% MLS; in strains from the USA, phenotypes were 33.3% iMLS, 33.3% iMLS-D, and 33.3% M. Erythromycin resistance genes in strains from Mexico were mefA (95%) and ermA (5%); USA strains harbored ermA (56%), mefA (33%), and none (11%). In Mexico, all erythromycin-resistant strains were non-invasive, whereas 89% of strains from the USA were invasive.

Conclusions: Erythromycin resistance continues to exist at low levels in both Mexico and the USA, although the genetic mechanisms responsible differ between the two nations. These genetic differences may be related to the invasive character of the \textit{S. pyogenes} isolated.

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1. Introduction

\textit{Streptococcus pyogenes}, or group A streptococci (GAS), is one of the most important human pathogens. It has been associated with both non-invasive infections, such as acute pharyngitis, and invasive infections, such as cellulitis, necrotizing fasciitis, bacteremia, sepsis, and toxic shock syndrome.\textsuperscript{1}

GAS remains very sensitive to penicillin in vitro,\textsuperscript{2} and this antibiotic is the drug of choice in the treatment of most streptococcal infections because of its narrow spectrum, safety, low cost, and its efficacy in the prevention of rheumatic fever. For patients allergic to penicillin, erythromycin and other macrolides are used. For those patients with serious soft tissue infections, clindamycin is the preferred treatment because of its ability to inhibit the production of streptococcal virulence factors, including capsule, M protein, and exotoxins such as NADase and pyrogenic exotoxin A (SpeA). Clindamycin has also been shown to modulate both the promitogenic activity of SpeA\textsuperscript{3} and the host response to infection.\textsuperscript{4}

Erythromycin inhibits RNA-dependent protein synthesis in GAS. Different mechanisms of macrolide resistance in GAS have
been described. An efflux system for resistance to 14- and 15-membered, but not 16-membered, ring macrolides is encoded by the mefA or the mefO genes (transposable elements). GAS possessing this system are said to be of the M phenotype and are characterized by resistance to erythromycin and susceptibility to clindamycin.\textsuperscript{3-5} Another mechanism is decreased binding of erythromycin, other macrolides, lincosamides and streptogramin type B (MLSB phenotype) to their targets on the ribosome due to an altered conformation of a methylase enzyme encoded by two classes of genes, ermA and ermB subclass TR. MLSB resistance can be either inducibly (imLSB phenotype) or constitutively (cMLS B phenotype) expressed.

Since the 1990s, macrolide resistance has increased worldwide and ranges between 5% and 78%, with the highest prevalence in Asia. In America and Europe, the prevalence of macrolide resistance in GAS strains is around 5%.\textsuperscript{6}

The comparative prevalence, the associated phenotypes, and the genes responsible for erythromycin resistance in GAS isolates from invasive and non-invasive infections in Mexico and the USA are unknown. Thus, the aim of the present study was to compare these characteristics in such GAS isolates from these neighboring countries.

2. Materials and methods

2.1. Strains

We studied 467 GAS strains from Mexico and 174 GAS strains from USA. Strains were identified as GAS by colony morphology, the presence of \( \beta \)-hemolysis when plated on 5% sheep blood agar, sensitivity to bacitracin (0.04 \( \mu \)g; TAXO A; BBL Becton Dickinson, Sparks, MD, USA), and agglutination by specific antisera (Slide Strep To A; bioMérieux).

Susceptibility to penicillin (10 \( \mu \)g), erythromycin (15 \( \mu \)g), clindamycin (2 \( \mu \)g), ceftriaxone (30 \( \mu \)g), and vancomycin (30 \( \mu \)g) disks (BBL Becton Dickinson, Sparks, MD, USA) was performed on Mueller–Hinton 5% sheep blood agar plates (MHSBA) (BBL Becton Dickinson, Sparks, MD, USA) by the Kirby–Bauer method. Inhibitor diameter zones were interpreted in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI; M100-S17, 2010).\textsuperscript{9} Streptococcus pneumoniae ATCC 49619 was used for quality control. Strains that showed resistance to erythromycin using this method were subjected to (1) a micro-broth dilution test according to the CLSI\textsuperscript{10} to establish antibiotic minimum inhibitory concentrations (MIC), and (2) the triple disk test (erythromycin, clindamycin, and spiramycin) placed on MHSBA in a triangular manner with 20 mm of separation between each other, to determine the phenotype of such resistance.\textsuperscript{11}

2.2. Detection of mefA, ermB, and ermTR genes

All erythromycin-resistant and intermediate strains were screened for the presence of resistance-related genes. Isolates were grown for 18 h on MHSBA plates at 37°C and 5% \( \text{CO}_2 \). A full loopful of each strain was suspended in 50 \( \mu \)l of lysis buffer (Tris–HCl, pH 7.6; NaCl, 0.5 M EDTA), boiled at 100°C for 5 min, cooled to room temperature for 15 min, centrifuged at 13,000 \( \times \) g for 5 min, and the supernatant recovered for PCR as DNA template. Primers were as follows: for mefA, 5’-AGT ATC ATT AAT CAC TAG TGC-3’ and 5’-TTC TTC TGC TAC TAA AAG TGC-3’; ermB, 5’-CAA GTG AAA AAG TAC TCA ACC-3’ and 5’-ACT AAC GTT ACT TAA ATT TTG CAG-3’; ermTR, 5’-ATA GAA ATT GGC TCA GGA AAA GC-3’ and 5’-CCC TGT TTA CCC ATT TAT AAA CG-3’.\textsuperscript{12} Amplification was performed in a DNA thermal cycler (GeneAmp, PCR System 9700; Applied Biosystems) with the following conditions: For mefA and ermTR genes: one cycle at 94°C for 3 min; 30 cycles at 94°C for 30 s; 50°C for 45 s; 72°C for 90 s; and one cycle at 72°C for 7 min. For ermB gene: one cycle at 94°C for 3 min; 30 cycles at 94°C for 30 s; 45°C for 45 s; 72°C for 90 s; and one cycle at 72°C for 7 min. Amplification products were run on 1% agarose gels (100 V for 40–60 min) and stained with ethidium bromide. The product sizes of the ermTR, ermB, and mefA genes were 500, 652, and 376 bp, respectively (Figure 1).

3. Results

The 467 GAS strains from Mexico were from six states (Jalisco, Durango, Aguascalientes, Morelos, Colima, and Nuevo Leon). The 174 GAS strains from the USA were from 23 states (Idaho, Tennessee, Virginia, Minnesota, Montana, Georgia, Washington, Utah, California, New York, Alabama, Texas, Alaska, Mississippi, Massachusetts, Oregon, District of Columbia, Connecticut, Wyoming, Maryland, Nevada, South Carolina, and North Carolina).

Of the strains from Mexico, all were isolated during the period 1999–2009 from patients ranging in age between 1 and 82 years (median 8 years). The sites of isolation were as follows: 271/467 (58%) were from patients with acute pharyngitis, 89/467 (19%) were from normally sterile sites (invasive), 57/467 (12%) were from other non-sterile sites, and 50/467 (11%) were from pharyngeal carriers.

Of the strains from the USA, all were isolated during the period 1999–2010 from patients aged between 5 and 91 years (median 41 years). In contrast to the GAS from Mexico, most of the USA strains – 148/174 (85%) – were isolated from normally sterile sites (invasive) (Table 1).

All strains were susceptible to penicillin, ceftriaxone, and vancomycin. A single strain from Mexico was resistant to clindamycin. Erythromycin resistance defined as a MIC \( \geq 1 \mu g/ m l \) by micro-broth dilution testing\textsuperscript{9} was found in 23 (4.9%) Mexican strains and nine (5.2%) USA strains (Table 1). Phenotypes of erythromycin-resistant strains identified in Mexico were M.
Table 1
Demography and general characteristics of the GAS strains studied from patients with both invasive and non-invasive infections from Mexico and the USA

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mexico</th>
<th>USA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total of GAS strains</td>
<td>467</td>
<td>174</td>
</tr>
<tr>
<td>Patients age range (years)</td>
<td>1–82 (median 8 years)</td>
<td>5–91 (median 41 years)</td>
</tr>
<tr>
<td>Invasive</td>
<td>89/467 (19%)</td>
<td>148/174 (85%)</td>
</tr>
<tr>
<td>Erythromycin resistance</td>
<td>23/467 (4.9%)</td>
<td>9/174 (5.2%)</td>
</tr>
<tr>
<td>Phenotypes</td>
<td>M (95%); cMLS (5%)</td>
<td>iMLS (33.3%); iMLS-D (33.3%); M (33.3%)</td>
</tr>
<tr>
<td>Erythromycin resistance-related genes</td>
<td>mefa (9%); ermB (5%)</td>
<td>ermTR (56%); mefa (33%); none (11%)</td>
</tr>
</tbody>
</table>

GAS, group A Streptococcus.

Figure 2. Phenotypes identified among erythromycin-resistant group A Streptococcus strains isolated from patients with invasive and non-invasive infections; Mexico and USA.

Figure 3. Genes identified among erythromycin-resistant group A Streptococcus strains isolated from patients with invasive and non-invasive infections; Mexico and USA.

(linked to mefa) 95% and cMLS (linked to ermB) 5%. In strains from the USA, phenotypes were iMLS (linked to ermTR) 33.3%, iMLS-D (linked to ermTR) 33.3%, and M (linked to mefa) 33.3% (Figure 2). Genes detected in erythromycin-resistant strains from Mexico were mefa (95%) and ermB (5%). Strains from the USA harbored ermTR (56%) and mefa (33%); no erythromycin resistance-related genes were found in one strain out of the nine studied (i.e., 11%) (Figure 3). In Mexico, all erythromycin-resistant strains belonged to the non-invasive group, whereas 89% of strains from the USA were from invasive cases.

4. Discussion

Since the first description of erythromycin resistance in 1955, several reports have demonstrated a progressive increase in the prevalence of erythromycin resistance worldwide; this increase has been attributed to the use of erythromycin and other macrolides in the population. According to a review of trends in antibiotic utilization in eight Latin American countries, Mexico ranked fifth in the consumption of macrolides, lincosamides and streptogramins during 1997–2007. Despite this, the prevalence of erythromycin-resistant strains isolated from 1999 to 2010 (4.9%) was similar to the prevalence among strains from the USA (5.2%) during almost the same period of time. This prevalence was also similar to that reported in the USA during 2002–2003 (6–8%)

Regarding the phenotypes isolated among GAS strains from both Mexico and the USA, the M phenotype was predominant (95% and 100%, respectively) in GAS strains isolated from non-invasive infections and carriers. However, discordance between invasive GAS isolates was marked between the two countries. Specifically, 19% (n = 89) of Mexican GAS strains were classified as invasive; however none showed resistance to erythromycin. In contrast, 6% of 148 invasive GAS from the USA demonstrated erythromycin resistance. These erythromycin-resistant USA isolates were almost exclusively of the inducible MLS phenotype (iMLS and iMLS-D; 7%). In total, these findings agree with other studies from the USA, Italy, France, Mexico, Germany, India, and Austria that have also reported a predominance of the M phenotype in non-invasive isolates, and all the cited studies except the Mexican report have described the iMLS phenotypes in the invasive ones.

Our results also show that the mefa gene (always associated with the M phenotype) was the most prevalent gene associated with erythromycin-resistant strains isolated from patients with non-invasive infections in both countries. This is also consistent with other reports from elsewhere. The ermTR gene (associated with iMLS and iMLS-D phenotypes) was the most prevalent among GAS strains isolated from patients with invasive infections in the USA. This association was also seen in Italy and France. A single erythromycin-resistant strain from the USA that belonged to an iMLS-D phenotype, showed none of the studied erythromycin resistance-related genes. A possible explanation for the presence of erythromycin resistance besides mefa, ermB, and ermTR genes includes mutations of the 23S rRNA and the L4 ribosomal protein (not investigated).

In summary, (1) the prevalence of erythromycin-resistant GAS strains was low and comparable with other studies from Latin America and the USA; (2) the M phenotype (always associated with the mefa gene) was the most prevalent erythromycin-resistant phenotype associated with non-invasive isolates; and (3) invasive isolates demonstrating erythromycin resistance were only found in strains from the USA; these were predominantly associated with the inducible MLS phenotypes.

In conclusion, although the incidence of erythromycin resistance among GAS is comparable between Mexico and the USA, the mechanisms of erythromycin resistance are markedly different, suggesting that geographic location and the relative incidence of severe invasive GAS infection in the two countries influences the prevalent strain phenotype and genotype. Evidence from this study suggests that despite the close proximity of Mexico and the USA, erythromycin-resistant GAS strains are not sharing some erythromycin resistance genes. Since most (75%) of the erythro-
macin-resistant GAS strains isolated from patients with invasive infections in the USA also have inducible resistance to clindamycin, physicians should consider alternative antibiotics for such patients.

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Conflict of interest: All authors have no conflicts of interest to declare.

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