Gradient plate method to induce *Streptococcus pyogenes* resistance

Helene Carsenti-Etesse, Pierre-Marie Roger, Brigitte Dunais, Sophie Durgeat, G. Mancini, M. Bensoussan and Pierre Dellamonica

In recent years, increasing numbers of *Streptococcus pyogenes* (GAS) strains displaying resistance to macrolides have been reported in Finland, Japan, Asia and Spain. Antibiotic use has been shown to be a risk factor for infection with and carriage of drug-resistant streptococci. The aim of this study was to compare in-vitro development of resistance of streptococci to β-lactams (penicillin, amoxycillin, cefotiam and cefuroxime) and erythromycin by serial passages in subinhibitory concentrations of antibiotics (subMICs) by gradient plate method. Three clinical strains of GAS were tested. Two were susceptible to erythromycin (MIC = 0.015 mg/L and 0.013 mg/L) and one resistant. Serial passages were performed daily by gradient plate method until a four-fold increase of the MIC was achieved. GAS variants obtained after serial passages in β-lactams had MICs increased at least four-fold. They remained susceptible to these antibiotics. With erythromycin, final MICs reached intermediate and resistant level. Results obtained in this study with erythromycin are in good correlation with clinical studies showing that prior exposure to macrolides may help to facilitate the emergence of drug-resistant strains of streptococci.

**Introduction**

*Streptococcus pyogenes* (GAS) has been recognized as a significant human pathogen commonly associated with pharyngitis in children and cutaneous infection in individuals of all ages. In the pre-antibiotic era, GAS often caused serious invasive disease with a case fatality rate of up to 72%. Subsequently, mortality from GAS infections decreased substantially with a parallel decrease in the non-suppurative complications of streptococcal disease. Recently, a toxic shock-like syndrome associated with GAS has been recorded. These events suggest that important changes in the pattern of GAS infections may still occur. It is of major importance to avoid any resistance development in these isolates.

Penicillin is uniformly active against GAS and has remained the antibiotic of choice for the treatment of infections caused by this organism. Erythromycin or clindamycin has been recommended as an alternative treatment for patients allergic to penicillin. A low level of potency of erythromycin against a large proportion of isolates has been reported in Australia, Finland, the UK and Japan. It is possible that the widespread use of erythromycin is related to the emergence of isolates with elevated MICs. Therefore, knowledge concerning the ability of antibiotics commonly used in therapy to select streptococcal variants with decreased susceptibility is needed to promote rational antimicrobial use.

The aim of this study was to compare in-vitro development of resistance of streptococci to β-lactams and erythromycin by serial passages in subinhibitory concentrations of antibiotics by a gradient plate method.

**Material and methods**

Three clinical strains of *S. pyogenes* (Table I) were tested. Two strains were susceptible to erythromycin (MIC 0.015 mg/L and 0.013 mg/L) and one was resistant.
Antibiotics were supplied as laboratory standard powders of known potency. The antibiotics used were penicillin, amoxycillin (SmithKline Beecham, Paris, France), cefotiam (Takeda, Osaka, Japan), cefuroxime (Glaxo, Paris, France) and erythromycin (Abbott, Paris, France).

**MIC determinations**

MIC determinations were performed by two methods: Macrodilution method in brain heart broth was performed with bacterial inoculum $5 \times 10^5$ cfu/mL. MICs were defined as the lowest concentration of antibiotic resulting in no growth.

MICs were determined as described by Marty et al.\(^9\) by gradient plate method using a spiral inoculator:

$$\text{MIC} = (D \times R \times A)/h \times C.$$  

Where $C$ was defined as the initial concentration of antibiotic, $h$ was the agar thickness (in mm), $D \times R \times A$ was the antibiotic concentration at radial migration point deducted from the growth-inhibition diameter according to a specific table.

MICs were also determined by Etest at the beginning and after each seventh passage. A reference Staphylococcus aureus strain (ATCC 25293) was used as control for antibiotic solutions and Etest.

**Serial passages**

A continuous concentration gradient of each antibiotic was plated on to blood agar using a spiral inoculator.\(^{10}\) Plates were rested for at least 6 h to ensure diffusion of the antibiotic. Bacterial inoculum size was $10^6$ cfu/mL. MICs were determined after overnight incubation at $37^\circ C$ in a 5% CO\(_2\) atmosphere. Colonies nearest to the inhibition zone (cultured with subMICs) were selected using swabs and diluted to the inoculum size. This procedure was done daily until a significant increase of the MIC (by a factor of at least four) was obtained.

**Phenotypes of resistance**

Classification of resistance was based on the modified method of Seppälä et al.\(^6\) A sterile cotton swab was dipped into a bacterial suspension with an adjusted turbidity and was then streaked on to Mueller–Hinton agar supplemented with 5% sheep blood. The erythromycin disc (15 IU) and clindamycin disc (2 IU) (Pasteur, Paris, France) were placed 15 to 20 mm apart on the surface of the agar. The plate was incubated at $35^\circ C$ for 18 h. A blunting of the clindamycin inhibition zone toward the erythromycin disc was interpreted as inducible resistance. Resistance to clindamycin with no blunting of the clindamycin inhibition zone and erythromycin resistance indicated constitutive resistance. Resistance by efflux system (mef) was characterized by susceptibility to clindamycin and resistance to erythromycin.

**MIC breakpoints of oral amoxycillin and cephalosporins** were defined as: resistant MIC $\geq 2$ mg/L and susceptible MIC ($S$) $\leq 0.50$ mg/L (NCCLS, 1997).\(^{11}\)

### Results

Results obtained with the gradient method are in good agreement with MICs determined by macrobroth method and E test, within ± 1 dilution.

With penicillin as the selecting agent, the method did not allow sufficient increase of MICs to reach intermediate or resistant levels. Increase of MICs by factors of eight and four was observed for strains A1, and A2 and A3, respectively (Table II).

With amoxycillin as the selecting agent, MICs at the end of passages were increased by factors of eight, 32 and four for the two erythromycin-susceptible strains (A1 and A2) and the erythromycin-resistant strain (A3), respectively. At the end, strains were still sensitive to amoxycillin according to breakpoints. MICs were 0.04 mg/L, 0.20 mg/L and 0.009 mg/L, respectively (Table I).

With cefuroxime as the selecting agent, MICs of cefuroxime at the end of passage were increased by factors of two (strain A1) and four (strains A2 and A3) and were 0.006, 0.007 and 0.004 mg/L, respectively (Table I).

With cefotiam as the selecting agent an increase by a factor of eight was obtained after 59, 54 and 46 passages (Table II). Resistance level was not reached.

With erythromycin as the selecting agent, an increase by factor of eight was obtained after 56 passages with strain

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**Table I**. MICs for *S. pyogenes* initial strains susceptible to erythromycin and mutant strain at the start and end of serial passages

<table>
<thead>
<tr>
<th>Antibiotics for selection</th>
<th>Penicillin</th>
<th>A moxycillin</th>
<th>Cefotiam</th>
<th>Cefuroxime</th>
<th>Erythromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MICs (mg/L)</td>
<td>start</td>
<td>end</td>
<td>start</td>
<td>end</td>
<td>start</td>
</tr>
<tr>
<td>Strain A1</td>
<td>0.001</td>
<td>0.008</td>
<td>0.005</td>
<td>0.040</td>
<td>0.015</td>
</tr>
<tr>
<td>Strain A2</td>
<td>0.001</td>
<td>0.004</td>
<td>0.005</td>
<td>0.200</td>
<td>0.015</td>
</tr>
<tr>
<td>Strain A3</td>
<td>0.001</td>
<td>0.004</td>
<td>0.002</td>
<td>0.009</td>
<td>0.015</td>
</tr>
</tbody>
</table>

I, intermediate level; R, resistant level.
Streptococcus pyogenes resistance, induction by gradient plate method

A1. The final MIC was 1.33 mg/L, at resistant level \(^{11}\) or intermediate level \(^{12}\) according to various interpretations. For strain A 2, the MIC increased by a factor of 128 after 59 passages and the final MIC was 6 mg/L (resistance level was reached). A n erythromycin mutant of the A 2 strain showed an efflux-resistant phenotype (Figure 1).

Discussion

The only cephalosporins with breakpoints approved by the National Committee for Clinical Laboratory Standards \(^{11}\) are cefotaxime, ceftriaxone and cefuroxime. MIC breakpoints for erythromycin resistance were \(\geq 1\) mg/L. \(^{11}\) The Antibiogram Committee of the French Society for Microbiology have assigned MIC breakpoints for erythromycin susceptibility as \(\leq 1\) mg/L and resistance as \(> 4\) mg/L. \(^{12}\) For Streptococcus pneumoniae, penicillin resistance is defined as MIC > 1 mg/L and for other \(\beta\)-lactams as \(> 2\) mg/L. \(^{12}\)

A cording to these breakpoints, we were not able to induce resistance to \(\beta\)-lactam antibiotics.

These results are different to those obtained in previous studies with S. pneumoniae. \(^{13}\) For these strains intermediate level was reached with penicillin and amoxycillin, and intermediate or resistant level with cephalosporins. Two potential explanations might account for these results: either the MICs of these antibiotics for S. pyogenes are very low, 10-fold lower than for S. pneumoniae or, the PBPs of streptococcus group A do not become altered in a similar way to those of S. pneumoniae as might have been expected from the distant phylogenetic relationship between the two organisms. \(^{14}\)

Three functional classes of macrolide resistance mechanisms exist in pathogenic bacteria: those that modify the ribosome, which is the target of the antibiotic; those that modify the antibiotic itself; and those that affect the rate of transport of the antibiotic across the cell membrane. Target modification is conveyed by the action of a family of methyltransferase enzymes encoded by the erm \(^{15}\) (for erythromycin ribosome methylation) genes. A second gene has been shown recently to encode resistance by increasing the transport of 14- and 15-membered macrolides from cells of S. pyogenes. A very similar gene has been cloned from macrolide-resistant strains of S. pneumoniae. This gene family is designated mef (for macrolide efflux) and is believed to encode a hydrophobic membrane protein which uses the energy of the proton motive force to pump macrolides from the interior of the cell. \(^{16}\)

With erythromycin it was possible to reach intermediate and resistant level in the two initial strains that were susceptible to erythromycin. Similar selection by macrolides was observed with S. pneumoniae strains. \(^{10,17}\) The induced resistance was to erythromycin but not clindamycin and is related to an efflux mechanism (M-phenotype). \(^{16}\) The efflux gene mef \(A\) has been found in clinical strains of S. pyogenes \(^{18}\) that also have the M-phenotype while mef \(E\), an efflux gene with 90% homology with mef \(A\), has been

<table>
<thead>
<tr>
<th>Strain</th>
<th>Erythromycin</th>
<th>Cefotaxime</th>
<th>Ceftriaxone</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>9 - 21</td>
<td>53 - 63</td>
<td>24 - 48</td>
</tr>
<tr>
<td>A2</td>
<td>21 - 46</td>
<td>24 - 48</td>
<td>23 - 43</td>
</tr>
<tr>
<td>A3</td>
<td>22 - 46</td>
<td>23 - 46</td>
<td>13 - 32</td>
</tr>
</tbody>
</table>

\(^{7}\) Not reached.

\(^{8}\) Strain resistant to erythromycin not done.
identified in clinically resistant strains of \textit{S. pneumoniae}.\textsuperscript{20}

\textit{S. pyogenes} has been considered uniformly susceptible to penicillin. However, there have been reports of sporadic failures of penicillin treatment and 11.2\% of \textit{Streptococcus} group \textit{B} strains are of intermediate susceptibility. \textit{Streptococcus} group \textit{F} strains have intermediate susceptibility to cefotaxime in 54\% of cases and resistance in 36.4\% of cases in Asia. Epidemiological studies are still needed.\textsuperscript{20} The high incidence of penicillin resistance among viridans streptococci observed in Taiwan is similar to that reported among isolates in Africa and Spain.\textsuperscript{21}

The results reported from the USA (38\%), Spain (34.8\%) and Taiwan (53.3\%) indicate that the emergence of a high incidence of erythromycin-resistant strains has limited the value of macrolides for the prophylaxis of viridans streptococci infection in high risk populations.\textsuperscript{22}

In Taiwan, the decreased activity of erythromycin against \textit{S. pyogenes} is a serious problem.\textsuperscript{20} Erythromycin is commonly used there in primary care clinics and is readily available over the counter from pharmacists, without prescription. Furthermore, this drug is prescribed frequently as a first-line antibiotic for patients with upper respiratory tract infections in hospitals. In Finland, Seppälä et al.\textsuperscript{6} revealed that variations in the prevalence of erythromycin resistance did not correlate well with differences in erythromycin use at the community level. Our in-vitro study is the first on \textit{S. pyogenes} isolates showing that resistance via the efflux mechanism may be obtained by multiple steps in the presence of subinhibitory concentrations of erythromycin.

The results obtained in this study are in good correlation with clinical studies showing that exposure to macrolides may help to facilitate emergence of drug-resistant streptococci.\textsuperscript{22,23} Considering the dangers of the spread of these resistant strains, indications for antibiotic therapy should be reviewed in favour of antimicrobial agents with lower selective properties.

References


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