In-vitro susceptibility, tolerance and glycocalyx production in Streptococcus mutans

A. De la Higuera, A. Castillo, J. Gutiérrez, A. García-Mendoza and J. Liébana*

Department of Microbiology, Odontology and Medicine Sections, University Hospital, University of Granada, 18012 Granada, Spain

We studied the presence of high-level resistance to aminoglycosides, penicillin tolerance and glycocalyx production in 160 isolates of Streptococcus mutans. Susceptibility to amoxycillin, cefazolin, imipenem, erythromycin, clindamycin, vancomycin and teicoplanin was also investigated. Of the isolates analysed, 58.8% produced glycocalyx in vitro and 2.5% were penicillin-tolerant. High-level resistance to streptomycin was found in 16.3% of the isolates, but all were sensitive to all other antibiotics tested. We found no significant relationship between glycocalyx production and high-level streptomycin resistance, penicillin tolerance or antibiotic susceptibility, except for a greater susceptibility to clindamycin and vancomycin in isolates that produced glycocalyx. Although our findings reflect the clinically favourable pattern of susceptibility currently found in this species, the appearance in some isolates of resistance, tolerance and glycocalyx production should be investigated because of the risks involved in endocarditis caused by S. mutans.

Materials and methods

A total of 160 isolates of S. mutans were isolated from human saliva or supragingival plaque, and were identified in accordance with the criteria of Maiden et al.8

Susceptibility studies

Antibiotic susceptibilities were determined using a solid medium dilution method in accordance with NCCLS recommendations for streptococci.9 A moxycillin, cefazolin, erythromycin, clindamycin and vancomycin were from Sigma Chemical Co. (St Louis, MO, USA), imipenem from Merck Sharp & Dohme (Madrid, Spain) and teicoplanin from Merrell Dow (Madrid, Spain). A nitribiotics were used at concentrations of 0.003-1 mg/L, except for vancomycin and teicoplanin, which were used at concentrations of 0.007-2.0 mg/L.

Detection of high-level resistance to aminoglycosides

High-level resistance to aminoglycosides was tested by the solid medium macrodilution method. The antibiotics assayed were gentamicin, streptomycin, tobramycin, kanamycin and amikacin (Sigma), each of which was tested at concentrations of 500, 1000 and 2000 mg/L.

*Corresponding author. Departamento de Microbiología, Facultad de Medicina, Avenida de Madrid 11, 18012 Granada, Spain. Tel: +34-58-243549; Fax: +34-58-243547.

© 1997 The British Society for Antimicrobial Chemotherapy.
Penicillin tolerance

Tolerance to penicillin was detected by the liquid medium macrodilution method in Todd–Hewitt broth (Oxoid, Basingstoke, U.K.) We tested ten concentrations of penicillin (Sigma) ranging from 0.003 to 2 mg/L. The inoculum consisted of approximately $5 \times 10^5$ cfu/mL from a suspension of a bacterial inoculum at each concentration plus 1 mL of bacterial inoculum were incubated at 36 ± 1°C for 24 h, and the MIC was determined. The MBC was then found by the method of James. A 0.1 mL volume from each tube showing no evident growth was transferred on to 1% horse blood agar plates (frozen–thawed laked erythrocytes) and 0.5 U/mL β-lactamase (Sigma). The plates were incubated for 24 h at 36 ± 1°C and the MBC was determined.

Glycocalyx production

Qualitative assays were used with all 160 isolates of S. mutans to detect the production of glycocalyx in vitro, according to the method of Molisch. Bacteria from Wilkins-Chalgren agar (Oxoid) were inoculated in 4 mL of fetal bovine serum (Bio Whittaker, Heidelberg, Germany), incubated for 48 h at 36 ± 1°C and then harvested by centrifugation at 950g for 10 min. The pellet was resuspended in 4 mL of 0.9% saline solution and gently sonicated for 30 s. After centrifugation, two drops of α-naphthol (Sigma) diluted to 10% with absolute ethanol were added to 0.5 mL of supernatant and 0.5 mL of sulphuric acid (Panreac, Barcelona, Spain). Tests were considered positive when a purple–red interface appeared between the two solutions. A s a negative control we used a strain of Proteus mirabilis known to be a non-producer of glycocalyx. S. mutans strain O M Z 176 (now Streptococcus sobrinus) was used as a positive control.

Statistical analyses

The results were compared using the chi-squared test and analysis of variance, by means of the R-Sigma statistical program (Horus Hardware, Madrid, Spain). All assays were repeated several times to check the consistency of the results.

Results

The MIC$_{50}$, MIC$_{90}$ and mean MIC values for the antibiotics tested are shown in the Table for the 160 isolates of S. mutans. In all isolates, the MICs were lower than the breakpoint for resistance. Only ten isolates showed reduced sensitivity to amoxicillin (four with a MIC of 0.25 mg/L and six with a MIC of 0.5 mg/L). Two isolates showed reduced sensitivity to erythromycin and clindamycin (MIC = 1 mg/L).

We found no high-level resistance to gentamicin, tobramycin, kanamycin or amikacin at any of the concentrations tested. However, 26 isolates (16.3%) were resistant to streptomycin at 500 mg/L, 16 (10%) at 1000 mg/L and eight (5%) at 2000 mg/L.

Four isolates of S. mutans (2.5%) were penicillin-tolerant. Two of these had an MIC of 0.03 mg/L and an MBC of ≥1 mg/L; in the other two, the MIC was 0.25 mg/L and the MBC ≥8.0 mg/L.

Glycocalyx production was found in 94 of the 160 isolates (58.8%). A nalysis of variance failed to substantiate a significant relationship between glycocalyx production and MIC for most antibiotics, except for increased susceptibility to clindamycin and vancomycin (P < 0.001 in both cases). Likewise, chi-squared tests failed to detect any significant relationships between the results for the different parameters.

Discussion

The initiation and maintenance of subacute endocarditis is influenced by several characteristics of the microorganism involved, such as its capacity to produce glycocalyx, susceptibility to antibiotics, high-level resistance to aminoglycosides and penicillin tolerance. This makes it necessary to identify these features in S. mutans, a species often involved in such infections. As S. mutans is the main pathogen found in connection with dental caries and is present in the oral cavity in most humans, the risk of bacteraemia caused by this microorganism after dental procedures is high.

Aminoglycosides, especially streptomycin and gentamicin, are frequently used to treat endocarditis caused by viridans streptococci. These antibiotics are often used in conjunction with penicillin because of the synergic effect found both in vitro and in vivo. Other aminoglycosides, such as tobramycin or kanamycin, are rarely used to treat endocarditis, because they show no greater synergic effects when combined with penicillin.

Different studies have used different breakpoints for

**Table.** MIC (mg/L) of several antibiotics for 160 isolates of S. mutans

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>R range</th>
<th>Mean</th>
<th>MIC$_{50}$</th>
<th>MIC$_{90}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A moxycillin</td>
<td>0.003–0.5</td>
<td>0.06</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>0.007–1</td>
<td>0.08</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.007–1</td>
<td>0.08</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.007–1</td>
<td>0.07</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.003–1</td>
<td>0.09</td>
<td>0.01</td>
<td>0.17</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.03–1</td>
<td>0.46</td>
<td>0.34</td>
<td>0.73</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>0.06–1</td>
<td>0.56</td>
<td>0.38</td>
<td>0.85</td>
</tr>
</tbody>
</table>
high-level resistance, namely \( \geq 500 \) mg/L, \( \geq 1000 \) mg/L or even \( \geq 2000 \) mg/L.\textsuperscript{4,7} Although it is difficult to define the concentration at which high-level resistance to a particular antibiotic appears, it is advisable to use as a breakpoint the MIC at which synergy between penicillin and the aminoglycoside disappears and therapeutic effectiveness is lost.\textsuperscript{4}

The appearance of high-level resistance to aminoglycosides is usually mediated by acetyl-, adenyl- or phosphotransferase enzymes that modify the antibiotic.\textsuperscript{7,14} Although this mechanism of resistance is the most widespread, a ribosomal mechanism of streptomycin resistance has also been described and found to depend on a single aberrant protein (S12) of the smaller ribosomal subunit.\textsuperscript{15,16}

The rates of high-level resistance to streptomycin we found in S. mutans did not differ significantly from those in earlier reports of viridans streptococci, in which resistance was detected in 2–7.9% of the isolates tested.\textsuperscript{4,17} The absence of cross-resistance to other aminoglycosides among the isolates we tested suggests that resistance may have arisen via the adenyltransferase pathway, which inactivates only streptomycin, as reported in Enterococcus spp.\textsuperscript{7}

The term ‘tolerance’ is used when the MBC is significantly higher than the MIC (MBC/MIC ratio \( \geq 32 \)).\textsuperscript{18,19} Tolerant bacteria are killed more slowly than non-tolerant cells with the same MIC.\textsuperscript{18} Tolerance to \( \beta \)-lactams is related to a deficit in murine hydrolase, an enzyme involved in processes of bacterial lysis that take place after the \( \beta \)-lactam antibiotic has bound to penicillin-binding proteins on the bacterial wall.\textsuperscript{5,18,20,21} This phenomenon can be studied in vitro, although it is important to develop standardized methods to ensure reproducibility of the results, and to facilitate comparisons between studies.\textsuperscript{9,21}

Tolerance is especially widespread among streptococci from gingival plaque and in blood cultures of samples obtained after dental extractions.\textsuperscript{22} Tolerant strains have also been reported in Spain among bacteria that cause endocarditis.\textsuperscript{18} The percentages of tolerant strains are largest in Streptococcus sanguis, especially in type I,\textsuperscript{23} Streptococcus mitior (currently designated Streptococcus mitis) and S. mutans, which are the species most frequently involved in the development of endocarditis.\textsuperscript{19} Although the appearance of tolerance was initially thought to have no clinical repercussions,\textsuperscript{24} it may be associated with the failure of penicillin treatment in diseases such as endocarditis.\textsuperscript{9,20} Tolerant isolates show weaker responses to penicillin, even when it is administered in conjunction with streptomycin.\textsuperscript{25,26} Penicillin tolerance is also a factor in the failure of prophylaxis with a single dose of amoxycillin in patients at risk of developing endocarditis.\textsuperscript{5,19,26}

Of the 160 isolates we studied, only four (2.5%) had an MBC/MIC ratio of \( >32 \); this proportion is lower than the figures given in earlier studies.\textsuperscript{5,22,27}

Most streptococci involved in subacute endocarditis are sensitive to antibiotics commonly used both for prophylaxis and for treatment.\textsuperscript{28–30} \( \beta \)-Lactams have classically constituted the basis of therapy against this infection, as the causal organisms continue to show excellent levels of susceptibility to these antibiotics.\textsuperscript{17,28} A nether group of drugs also found to be effective against subacute endocarditis comprises bacteriostatic agents such as erythromycin and clindamycin, which are used in prophylaxis against bacteraemia after dental extraction.\textsuperscript{31,32} In contrast with drugs that lead to bacterial lysis, bacteriostatic agents are thought to prevent bacteraemia by inhibiting the adherence of streptococci to the heart valves.\textsuperscript{33,34} Vancomycin and teicoplanin, used in people who are allergic to penicillin, are useful in both the prophylaxis and treatment of endocarditis.\textsuperscript{35,36} Imipenem and other carbapenems are active both alone and in combination with aminoglycosides in the treatment of streptococcal endocarditis and may therefore constitute an alternative in patients who are allergic to penicillin.\textsuperscript{36–39} These drugs may also be useful in hospitalized patients with endocarditis.

S. mutans is usually one of the most sensitive of all oral streptococci to antibiotics, both in isolates from dental plaque and in patients with infectious endocarditis.\textsuperscript{28,40,41} The results of our susceptibility tests were similar to those found by other authors for oral streptococci. Our data were also in agreement with the findings of earlier studies in our setting, which showed that clindamycin was more effective than erythromycin against S. mutans, and support the choice of the former antibiotic in the prophylaxis against streptococcal endocarditis.\textsuperscript{29,30}

A crucial aspect of the pathogenesis of endocarditis caused by viridans streptococci is the interaction between the bacteria and the damaged heart valve. The ability of some members of this group, notably S. mutans, S. sanguis and S. mitis, to adhere to the extracellular matrix of the cardiac endothelium partly accounts for the high incidence of endocarditis these species cause.\textsuperscript{5,42} This trait is associated with high levels of glyocalyx production.\textsuperscript{42} Streptococci able to form large amounts of glyocalyx form larger vegetations than strains without this ability;\textsuperscript{42} this may be a direct consequence of the larger numbers of bacterial cells adhering to the valvular surface, or to increased platelet and fibrin deposition.\textsuperscript{44} Within the vegetation there are two populations of bacteria: resting cells, located more deeply, and growing cells, located superficially. Only the latter population is susceptible to treatment with \( \beta \)-lactams and aminoglycosides; hence, the thicker the vegetation, the more difficult it is to eliminate with antibiotic treatment.\textsuperscript{45} Moreover, the formation of large cardiac vegetations makes it difficult for the antibiotic to penetrate them fully and also interferes with the host’s defence mechanisms (e.g., complement, antibodies and phagocytic cells). This makes the lesion more likely to persist and makes cure more difficult.\textsuperscript{9,43,45,46}

The in-vitro production of glyocalyx reflects the ability of a strain to produce this component in the living heart and detection of this ability is thus a potentially valuable
predictor of pathogenicity. Strains that cause endocarditis vary in the amount of exopolysaccharide they produce; moreover, qualitative as well as quantitative differences in the glyocalyx have been found between streptococci that cause the disease and species not associated with this infection. We detected glyocalyx formation in 58.8% of the isolates tested. However, because the glyocalyx may be lost in the course of subculture, the in-vivo figure may be higher. Statistical analyses confirmed earlier findings that suggested no difference in susceptibility in vitro (except for clindamycin and vancomycin) between isolates that produce exopolysaccharide and those that do not. Failure of treatment was not related to changes in MIC, but rather to lower accessibility of the vegetation to the antibiotic in the cardiac focus of infection. The apparent association of glyocalyx production with susceptibility to clindamycin and vancomycin is hard to explain, although it may have been due to chance.

In conclusion, we found that S. mutans was susceptible to antibiotics commonly used in the prophylaxis and treatment of endocarditis. The frequencies of penicillin tolerance and high-level resistance to aminoglycosides among the isolates we investigated were low. However, the large percentage of isolates able to produce glyocalyx in vitro may make this microorganism difficult to eradicate in infectious endocarditis.

Acknowledgements

This study was partially supported by the Andalusian Regional Government through the research project ‘Microbiology, Immunology and Epidemiology of Oral Diseases’. We thank Karen Shashok for translating the original manuscript into English.

References

Susceptibility and glycoalyx production in S. mutans


Received 7 November 1996; returned 4 February 1997; revised 6 March 1997; accepted 21 April 1997