Introduction

Drug-resistant tuberculosis has become a serious challenge to control programmes. Multidrug-resistant (MDR) tuberculosis, defined as tuberculosis caused by Mycobacterium tuberculosis strains resistant to at least isoniazid and rifampicin, poses a special problem since the patients infected with these strains cannot be cured by the standard WHO re-treatment regimen. Most second-line drugs used for the treatment of drug-resistant tuberculosis are either toxic or very expensive. There is an urgent need to search for effective anti-tuberculosis drugs which are affordable in low-income countries, where the situation is alarming. Penicillins have been widely used for the last five decades for the treatment of infections by Gram-positive and Gram-negative bacteria. Some β-lactam antibiotics, such as amoxycillin and ampicillin, have been tested on different clinically important species of mycobacteria including M. tuberculosis. Mycobacteria vary in their susceptibility to β-lactam antibiotics mainly due to variation in β-lactamase production. M. tuberculosis produces β-lactamase; the combination of β-lactam antibiotics such as amoxycillin with β-lactamase inhibitors such as clavulanic acid has been shown to be more effective than amoxycillin alone. The lipid-rich cell wall of mycobacteria is also a major factor in the relative resistance of mycobacteria to β-lactam antibiotics.

Materials and methods

Strains

A total of 30 strains of M. tuberculosis isolated from Danish and Ethiopian tuberculosis patients were included in this study. All isolates were identified with standard biochemical tests and an M. tuberculosis-specific DNA-RNA probe (Accu Probe; GenProbe Inc., San Diego, CA, USA). Ten strains were susceptible to all first-line antimycobacterial drugs, 20 strains were MDR and ten of these were also resistant to ethambutol. M. tuberculosis
H₃₇Rv (ATCC 25618) was used as a control in the susceptibility tests. M. tuberculosis fortuitum (ATCC 6841) was used as a control for the assay of β-lactamase activity, and in the disc diffusion experiments. Three clinical strains of M. avium avium were used as negative controls for the assay of β-lactamase activity.

Drugs

Stock solutions of amoxycillin (Beecham Pharmaceuticals, Worthing, U.K.), clavulanic acid (Beecham Pharmaceuticals) and ethambutol (Sigma, St Louis, MO, USA) were prepared in sterile phosphate-buffered saline (PBS) pH 6.8 at a concentration 40 times higher than the maximum final concentration needed in 4 mL of test medium. The stock solutions were kept at −20°C in small aliquots and new solutions were prepared every 2 weeks. Different working dilutions of the drugs were prepared on the day of the experiment.

Assay for β-lactamase activity

A semi-quantitative nitrocefin method was used to test for β-lactamase activity. A solution of nitrocefin (Oxoid, Basingstoke, U.K.) with a concentration of 500 mg/L was prepared by reconstituting lyophilized nitrocefin with a rehydration fluid provided by the manufacturer. A 200 μL aliquot of bacteria from a pellet of a 2 week old culture in Dubos broth was transferred into screw-capped tubes. Nitrocefin solution (40 μL) was added and the tubes were incubated at 37°C. The colour change was assessed after 1 h and after 24 h of incubation. A strain of M. fortuitum was used as a positive control and three strains of M. avium as negative controls. A tube containing only PBS and nitrocefin was used as a reagent control. The β-lactamase production was graded as (−) when there was no colour change, (+) when the red colour was less intense than the positive control and (+++) when the red colour was as intense as the positive control. All tests were done in duplicate.

Disc diffusion assay

M. fortuitum grown in Dubos medium was used to prepare a suspension in PBS corresponding to a McFarland 0.5 turbidity standard. A 1 in 10 dilution of this suspension was used for the disc diffusion assay. Blood agar was inoculated by streaking with a cotton swab moistened with the bacterial suspension. The blood agar was left to dry at 37°C for 15 min. Paper discs containing single drugs (4 μg ethambutol, 13 μg amoxycillin and 13 μg clavulanic acid) or combinations of drugs (13 μg amoxycillin and 13 μg clavulanic acid; 13 μg amoxycillin and 4 μg ethambutol; 13 μg amoxycillin, 13 μg clavulanic acid and 4 μg ethambutol) were placed uniformly on the plate which was incubated at 37°C. The diameter of the zone of inhibition around the paper discs was measured on the third day.

BACTEC susceptibility test

Susceptibility tests were performed with the BACTEC radiometric method (Becton-Dickinson, Sparks, MD, USA). Suspensions of bacteria were prepared by shaking 2 week old cultures in Dubos medium and adjusting the turbidity to that of a McFarland 0.5 standard with PBS. A 0.1 mL aliquot of the bacterial suspension was injected into BACTEC vials containing drugs or combinations of drugs. A bacterial suspension, diluted 100-fold, was prepared in PBS and 0.1 mL was injected into a vial containing no drug (control). All vials were incubated at 37°C and growth index (GI) values were recorded daily with the BACTEC-460 instrument. Interpretation of results were made according to the manufacturer’s instructions when the GI value of the control reached 30. Drug interaction was assessed using ‘X/Y quotients’ as described by Hoffner et al. Briefly, synergy was defined as X/Y < 0.5, where X is the GI value of the test vial containing amoxycillin, clavulanic acid and ethambutol, and Y is the lower GI value of vials containing amoxycillin and clavulanic acid or ethambutol alone. A quotient between 0.5 and 0.75 was interpreted as an additive interaction.

The MIC of ethambutol was determined for all isolates and H₃₇Rv. Final concentrations of 0.25, 0.5, 1.0, 2.0 and 4.0 mg/L were used for 20 isolates which were known to be susceptible to 5 mg/L ethambutol. Final concentrations of 2.5, 5 and 10 mg/L were used for ten isolates known to be resistant to either 2.5 or 5 mg/L ethambutol.

A moxycillin and clavulanic acid were tested alone at a concentration of 16 mg/L. The combinations of different concentrations of amoxycillin (1, 2, 4 and 8 mg/L) and 8 mg/L clavulanic acid were tested on all isolates. The combinations of different concentrations of amoxycillin (1, 2, 4 and 8 mg/L), 8 mg/L clavulanic acid, and a sub-inhibitory concentration of ethambutol were also tested on all isolates. For isolates with an MIC of ethambutol of ≤10 mg/L, the ethambutol used in the combinations was four times lower than the MIC. For six MDR isolates with an MIC of >10 mg/L, ethambutol 10 mg/L was used in combination studies. The inactivation of clavulanic acid at 37°C was compensated for by adding 4 mg/L every other day.

The effect of combinations of different concentrations of amoxycillin (0.5, 1 and 2 mg/L) and clavulanic acid on H₃₇Rv pretreated with a subinhibitory concentration of ethambutol (i.e. 3 days’ exposure to ethambutol at 0.25 × MIC) was also tested. The pretreatment with ethambutol was done in 5 mL Dubos broth and the bacterial turbidity was adjusted to that of a McFarland 0.5 standard with PBS after 3 days’ incubation. One hundred microlitres of the suspension was injected into BACTEC vials containing...
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different combinations of amoxycillin and clavulanic acid. The growth pattern of pretreated H$_{37}$Rv was compared with that of H$_{37}$Rv that had not been pretreated with ethambutol.

Results

Assay for β-lactamase activity

After incubation at 37°C for 1 h, 14/30 (47%) strains showed positive β-lactamase activity and 16/30 (53%) strains and H$_{37}$Rv showed strongly positive β-lactamase activity. All 30 isolates and the H$_{37}$Rv showed a strongly positive β-lactamase activity after 24 h incubation. The three M. avium strains included as negative controls showed no β-lactamase activity after 1 h but showed a strongly positive β-lactamase activity after 24 h incubation with nitrocefin. There was no colour change after incubation for 1 h and 24 h in a control tube containing PBS and nitrocefin.

Disc diffusion assay

Figure 1 shows the result of a disc diffusion assay. No zone of inhibition was seen around discs containing any of the single drugs (i.e. amoxycillin, clavulanic acid or ethambutol). The diameter of the zone of inhibition around a disc containing amoxycillin and ethambutol was larger (13 mm) than the zone around a disc with amoxycillin and clavulanic acid (9 mm). The maximum zone of inhibition (i.e. 18 mm) was seen around a disc containing all three drugs.

BACTEC susceptibility test

Figure 2 shows the MIC for ethambutol for 30 isolates. The MIC for H$_{37}$Rv was 2 mg/L. Of the 20 isolates (ten MDR and ten non-MDR) which were known to be susceptible to 5 mg/L ethambutol, 16 (80%) had an MIC of ≤ 2 mg/L. All ten MDR isolates that were known to be resistant to ethambutol had an MIC of ≥ 5 mg/L. Six of the ten resistant isolates had an MIC of > 10 mg/L.

The MIC of amoxycillin in combination with clavulanic acid and the effect of addition of a subinhibitory concentration of ethambutol on the MIC of amoxycillin are shown in the Table. All isolates were resistant to 16 mg/L amoxycillin as well as 16 mg/L clavulanic acid. In combination with clavulanic acid, the MIC of amoxycillin for 27/30 (90%) isolates was ≤ 2 mg/L. The MIC for MDR strains was no different from the MIC for the fully susceptible isolates. In the presence of clavulanic acid and a subinhibitory concentration of ethambutol, the MIC of amoxycillin for 29/30 (97%) strains was ≤ 0.5 mg/L. The interaction was synergic for 28/30 (93.3%) strains, with an X/Y quotient of 0.24 ± 0.125. The interaction of ethambutol with amoxycillin-clavulanic acid was additive for two (6.7%) of 30 strains, with an X/Y quotient of 0.6 in each case.

A subinhibitory concentration of ethambutol also had a marked effect in reducing the MIC of amoxycillin, even in the absence of clavulanic acid. Eighteen (60%) of the 30

Figure 1. Diffusion assay on M. fortuitum with paper discs containing single drugs and combinations of drugs. A, amoxycillin 13 μg; B, clavulanic acid 13 μg; C, ethambutol 4 μg; D, A + B; E, A + C; F, A + B + C.
isolates were susceptible to the combination of 8 mg/L amoxycillin and a subinhibitory concentration of ethambutol. The growth pattern of one of the strains in the presence of single drugs and some of the different combinations of drugs is shown in Figure 3.

The MIC of amoxycillin (with clavulanic acid) for H₃⁷Rv was 2 mg/L and the MIC was <0.5 mg/L when a subinhibitory concentration of ethambutol was added. A similar decrease in MIC was obtained with H₃⁷Rv pretreated with a subinhibitory concentration of ethambutol.

**Discussion**

MDR tuberculosis is increasing in many parts of the world and constitutes a special problem in low-income countries since most treatment regimens with a demonstrated activity against MDR strains are very expensive. Therefore, there is a need to search for antimycobacterial activity among established drugs, which are usually less expensive than newly developed drugs.

The disc diffusion assay is a simple way of studying interaction between antimicrobial drugs. In our initial experiments, a synergic interaction was demonstrated between amoxycillin, clavulanic acid and ethambutol on a strain of *M. fortuitum*. This finding was the basis for subsequent more extensive studies on strains of *M. tuberculosis*. We have shown that for 97% (29/30) of *M. tuberculosis* strains, the addition of ethambutol reduced the MIC of amoxycillin by four-fold or more, and that the MIC of amoxycillin in the presence of a subinhibitory concentration of ethambutol was <0.5 mg/L for all but one strain.

The MIC of amoxycillin/clavulanic acid alone is below

**Table.** MIC of amoxycillin in combination with 8 mg/L clavulanic acid alone and in combination with both clavulanic acid and a subinhibitory concentration of ethambutol for 30 strains of *M. tuberculosis*.

<table>
<thead>
<tr>
<th>Combination of drugs</th>
<th>No. (%) of isolates with MIC of amoxycillin (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>A moxycillin and clavulanic acid</td>
<td>1 (3.3)</td>
</tr>
<tr>
<td>A moxycillin, clavulanic acid and ethambutol</td>
<td>29 (96.7)</td>
</tr>
</tbody>
</table>

**Figure 2.** MIC of ethambutol for 30 clinical isolates of *M. tuberculosis*.

**Figure 3.** Pattern of *M. tuberculosis* growth in the presence of different drugs and combination of drugs. ◆, amoxycillin 16 mg/L; ■, ethambutol at 0.25 × MIC; ▲, amoxycillin 8 mg/L plus ethambutol at 0.25 × MIC; ○, amoxycillin 2 mg/L plus clavulanic acid 8 mg/L; □, amoxycillin 0.5 mg/L plus clavulanic acid 8 mg/L plus ethambutol at 0.25 × MIC. The undiluted control and the vial with clavulanic acid 16 mg/L had GI values similar to the vial with amoxycillin 16 mg/L.
the concentration achievable in serum.\textsuperscript{15,16} The further decrease in the MIC of amoxycillin through the synergic effect with ethambutol could be crucially important for greater activity at the site of the tuberculous infection, where it is difficult to achieve effective drug concentrations. Brun et al.\textsuperscript{16} reported that after a 1 500 mg oral dose of amoxycillin the concentration in the lung tissue ranges from 0.64 to 5 \mu g/g. This tissue concentration could be significantly increased by administering a higher dose of amoxycillin.\textsuperscript{16} The synergy with ethambutol is particularly important in MDR tuberculosis patients since clinical resistance to ethambutol is uncommon.\textsuperscript{1} The concentration of ethambutol achievable in serum is 2–6 mg/L after a single dose of 25 mg/kg and this concentration could be high enough to achieve a synergic effect even in ethambutol-resistant strains.

\textit{M. tuberculosis} produces broad-spectrum \beta-lactamases which inactivate both penicillins and cephalosporins.\textsuperscript{5} It has been reported that mycobacterial species such as \textit{M. tuberculosis} complex, \textit{Mycobacterium kansasii} and rapid growers are \beta-lactamase-positive while \textit{M. avium} complex are \beta-lactamase-negative.\textsuperscript{9} Similar results were obtained in our study when the nitrocefin test was interpreted after 1 h of incubation. However, all strains tested, including the \textit{M. avium} strains, were strongly positive after 24 h of incubation. This indicates that a much longer time than normally recommended might be needed to assess the production of \beta-lactamases in mycobacteria. It has been reported that clavulanic acid reduced the MIC of amoxycillin for almost 50% of the \textit{M. avium} complex strains tested,\textsuperscript{17} indicating a possible strain variation in the production of \beta-lactamase. More work is needed to evaluate the effect of the combination of \beta-lactams and \beta-lactamase inhibitors on MAC.

Pretreatment of \textit{M. tuberculosis} with sub-inhibitory concentrations of ethambutol significantly lowered the MIC of amoxycillin/clavulanic acid. This could be the result of a post-antibiotic effect of ethambutol and indicates that ethambutol does not have to be present with amoxycillin to exert its synergic effect. This could have pharmacokinetic implications and could favour a synergic effect of a single daily dose of ethambutol. A synergic interaction between ethambutol and some other \beta-lactam drugs such as cefazimid and cefepime on mycobacteria has been reported previously.\textsuperscript{18,19} Ethambutol also enhances the effect of many other drugs including aminoglycosides, rifamycins, quinolones and macrolides on mycobacteria.\textsuperscript{11,12} This key role may be explained by the effect of ethambutol on the integrity of the mycobacterial cell wall.\textsuperscript{20,21} We have investigated the possible effect of ethambutol on the binding of \textsuperscript{3}H-labelled benzyl penicillin to \textit{M. fortuitum} and on the production of \beta-lactamases (data not shown). However, we did not see any appreciable effect of pretreatment of \textit{M. fortuitum} with ethambutol either on the binding of \textsuperscript{3}H-labelled benzyl penicillin or on the production of \beta-lactamases. The actual mechanism by which ethambutol enhances the effect of \beta-lactams remains unclear and needs to be further investigated.

In the present study, the inactivation of clavulanic acid was compensated for by addition of fresh clavulanate every other day as recommended in the literature.\textsuperscript{14} However, the G1 values of vials where clavulanic acid was replenished and vials where clavulanic acid was not replenished were very similar (data not shown). This indicates that despite the short half-life of clavulanic acid at 37°C,\textsuperscript{14} the concentration of clavulanic acid usually used in combination studies (i.e. 8 mg/L) is high enough to inhibit \beta-lactamases at least until the fourth day, and there is no need to replenish if the susceptibility tests are run for less than a week.

Amoxycillin in combination with sulbactam has a bactericidal effect in vitro on exponential-phase cultures, indicating its usefulness in the early period of anti-tuberculosis treatment.\textsuperscript{8} Recent case reports have shown a possible effect of co-amoxiclav in the treatment of MDR tuberculosis.\textsuperscript{22,23} This should, in the light of the present study, be further evaluated in controlled treatment trials with regimens containing co-amoxiclav and ethambutol in combination with other antimycobacterial drugs.

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