Micro-broth dilution method with air-dried microplate for determining MICs of clarithromycin and amoxycillin for Helicobacter pylori isolates

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MICs of clarithromycin and amoxycillin for 253 isolates of Helicobacter pylori were measured by an air-dried microplate method and compared with the results obtained by the agar plate dilution method. The air-dried microplate method is performed by coating each well of a 96-well microplate with the test antibiotic and air-drying it. There were no marked differences between the air-dried microplate method and agar plate dilution methods in the MIC50 and MIC90 values or MIC ranges of clarithromycin obtained for the 253 isolates of H. pylori. More specifically, the MICs of clarithromycin for 114 (45.1%) of the 253 isolates were the same by the air-dried microplate method as the agar plate dilution method, and the differences in the MICs of clarithromycin for a further 114 isolates (45.1%) varied within one twofold dilution. The MICs of amoxycillin by the former method were in close agreement with the MICs obtained by the latter method: MICs of amoxycillin for 199 (78.7%) of the 253 isolates were the same by both methods, and the differences in the MICs of amoxycillin for 42 isolates (16.6%) varied within one twofold dilution. These results indicate that the air-dried microplate method is a useful method for determination of MICs, because the results obtained were in close agreement with those obtained by the standard agar plate dilution method. The air-dried microplate method is, therefore, a convenient and reliable method for determining the MICs of clarithromycin and amoxycillin for H. pylori isolates.

INTRODUCTION

Helicobacter pylori is the major causative pathogen of chronic gastritis and peptic ulcer disease (Blaser, 1992; Dunn et al., 1997; Graham, 1989). Eradication of the pathogen is recommended for patients whose gastric mucosa is infected with H. pylori, and combination chemotherapy consisting of amoxycillin, either clarithromycin or metronidazole and a proton-pump inhibitor is now used worldwide as eradication therapy (Calvet et al., 2000; Lind et al., 1999; Treiber et al., 1998).

In Japan, a combination of two antimicrobial agents, amoxycillin and clarithromycin, and a proton-pump inhibitor is now the most common regimen for the treatment of H. pylori infection based on the National Health Insurance system’s reimbursement regulations. While antimicrobial therapy to eradicate H. pylori from the gastric mucosa has led to improvement or normalization of the pathology of chronic gastritis, serious problems have been encountered in the treatment of H. pylori infections. First, H. pylori often recurs after apparently successful treatment, presumably because of incomplete eradication (Kalach et al., 2001a), and second, resistant strains sometimes emerge during or after treatment (Kalach et al., 2001b). Consequently, successful treatment of H. pylori infection requires the performance of antimicrobial susceptibility testing of the causative pathogen in clinical microbiology laboratories during or after treatment and timely feedback of the results to clinical staff. MICs of antimicrobial agents for H. pylori are usually determined by the agar dilution method according to the guidelines established by the NCCLS (1999, 2000). Routine determination of the MIC of an antibiotic given to H. pylori-infected patients by this method is inconvenient in most laboratories because the serial dilution of the test antibiotic and distribution into each test medium is a time-consuming task. The air-dried microplate method uses microplates previously coated with serial dilutions of a test antimicrobial agent and was developed as an easy method of determining MICs.

In the present study, the ability and reliability of the air-dried microplate method for determining the susceptibility of H. pylori isolates to clarithromycin and amoxycillin were evaluated in comparison with the reference agar dilution method.
METHODS

Test strains. A total of 253 H. pylori isolates were isolated in 1999 from gastric biopsy specimens of patients with gastritis or peptic ulcer. After culturing the specimens in selective agar, as described previously (Kobayashi et al., 2001), the isolates were stored at −80°C in Brucella broth (Becton Dickinson) containing 10% DMSO and 10% horse serum until used.

Antimicrobials and MIC determinations. The susceptibility of H. pylori isolates to clarithromycin (Taisho Pharmaceutical Co.) and amoxycillin (Sigma) was determined by the two different methods. The results were expressed as MIC₅₀ values (at which 50% of the isolates were inhibited), MIC₉₀ values and MIC ranges of the two antibiotics for the 253 isolates.

Agar dilution method. The MICs of the test antibiotics for H. pylori were determined by the agar dilution method according to the guidelines established by the NCCLS (2000).

Micro dilution method. A micro broth dilution method was performed with the Eiken Chemical dry plates. Each well of a 96-well microplate was coated with twofold serial dilutions of clarithromycin or amoxycillin and air-dried (0.0015–3.2 μg). A saline suspension of test strain equivalent to 2.0 McFarland standard (containing 1 × 10⁷ to 1 × 10⁸ c.f.u. ml⁻¹), was prepared from a 72 h-old subculture of a blood agar plate. The suspension (0.5 ml) was put into 9.5 ml cation-adjusted Mueller–Hinton broth (Difco) supplemented with 5% horse serum to a density of 5 × 10⁵–5 × 10⁶ c.f.u. ml⁻¹. A 100 μl volume of the suspension was added to each well, and the cultures were incubated at 35°C for 3 days under a microaerophilic atmosphere (O₂, 10%; CO₂, 5%). The MIC was defined as the lowest concentration of a test antibiotic that completely inhibited visible bacterial growth.

Quality control. H. pylori ATCC 43504 was used as the quality control strain, and MICs were determined by the above methods.

RESULTS AND DISCUSSION

The susceptibility of 253 H. pylori isolates to clarithromycin and amoxycillin is shown in Table 1. The MIC₅₀ and MIC₉₀ values, and MIC ranges for clarithromycin were almost the same by the air-dried microplate method and reference agar dilution methods: 0.06, > 32 and ≤ 0.015–32 μg ml⁻¹, and 0.06, 32 and ≤ 0.015–32 μg ml⁻¹, respectively. The MIC values for amoxycillin obtained by the two methods were nearly the same, and no, or only slight, differences were noted in their MIC₅₀ values, MIC₉₀ values and MIC ranges by the air-dried microplate method: ≤ 0.015, 0.12 and ≤ 0.015–1 μg ml⁻¹, respectively, and by the agar dilution method: ≤ 0.015, 0.06, and ≤ 0.015–1 μg ml⁻¹.

The MICs of clarithromycin for 114 (45.1%) of the 253 isolates determined by the two methods were the same, and the MICs for a further 114 isolates (45.1%) varied within a twofold dilution, showing close agreement in 90.1% of the test isolates. The MICs of amoxycillin for 199 (78.7%) of the 253 isolates were the same by the two methods, and the differences between the MICs for 42 isolates (16.6%) varied within a twofold dilution. The rate of agreement between the MICs of amoxycillin determined by the two methods was higher than the rate for clarithromycin reported above (Table 2).

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC (μg ml⁻¹)</th>
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<tr>
<td></td>
<td>MIC₅₀</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td></td>
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<tr>
<td>Air-dried microplate</td>
<td>≥ 0.015</td>
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<tr>
<td>Agar dilution</td>
<td>≥ 0.015</td>
</tr>
<tr>
<td>Amoxycillin</td>
<td></td>
</tr>
<tr>
<td>Air-dried microplate</td>
<td>≥ 0.015</td>
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<tr>
<td>Agar dilution</td>
<td>≥ 0.015</td>
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</table>
Table 2. Comparison of the MICs of 253 H. pylori isolates for clarithromycin and amoxycillin determined by the air-dried microplate method and the reference agar dilution method

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Air-dried microplate MIC/agar dilution MIC (log₂)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>−3</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>1</td>
</tr>
<tr>
<td>Amoxycillin</td>
<td>0</td>
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</table>

To compare the reliability of the MICs determined by the air-dried microplate method with those determined by the reference agar plate dilution method, the MICs of clarithromycin and amoxycillin for a reference strain of H. pylori, ATCC 43504⁴, were determined five times by both methods. The MICs for clarithromycin and amoxycillin determined by the air-dried microplate method were 0·03–0·06 µg ml⁻¹ and 0·03 µg ml⁻¹, respectively, while the MICs for clarithromycin and amoxycillin determined by the agar dilution method ranged from 0·03 to 0·12 µg ml⁻¹ and from ≈0·015 to 0·03 µg ml⁻¹, respectively.

Since 1999, the MICs of test antibiotics for H. pylori isolates have usually been determined by the agar dilution method according to the guidelines established by NCCLS M100-S9 (NCCLS, 1999). However, since H. pylori is fastidious and very slow-growing, it takes 3-4 days to form visible colonies for MIC determination under a microaerophilic atmosphere, and the resulting MIC values are greatly affected by the environmental conditions, including the CO₂ concentration. The MIC values of macrolide antibiotics for H. pylori isolates in particular are significantly affected by the presence of CO₂ because of acidification of the test medium (Debets-Ossenkopp et al., 1995). We previously reported two methods of resolving this problem: a semi-solid agar method that does not require a CO₂ atmosphere (Kobayashi et al., 1997) and an AnaeroPack method that generates a low CO₂ atmosphere (Kobayashi et al., 2001). In the guidelines established by the NCCLS (1999), the MIC interpretative standard of clarithromycin for H. pylori is defined as: susceptible, < 0·25 µg/ml⁻¹; intermediate resistant, 0·5-2·0 µg/ml⁻¹; resistant, > 2·0 µg/ml⁻¹; and the criteria for breakpoints of antimicrobial susceptibility for H. pylori are very narrow. As a result, a more precise method for determining the susceptibility of H. pylori is needed. The air-dried microplate method has been used as a convenient method for determination of MICs of antibiotics for various types of bacteria in some clinical microbiology laboratories in Japan, and it is expected to become extensively used as a reliable and convenient method of determining the MICs for H. pylori.

On the other hand, antimicrobial therapy with clarithromycin or metronidazole has failed to eradicate H. pylori in some patients because of the development of resistance to these antibiotics, which resulted in the recurrence of infection (Kalach et al., 2001a). Timely determination of the susceptibility of the pathogen isolated from the gastric mucosa of patients during or after treatment is the first step in ensuring successful therapy. However, the application of the reference agar dilution method for routine determination of the MICs of an antibiotic given to patients is inconvenient in most clinical microbiology laboratories.

In the present study, the MICs of clarithromycin and amoxycillin for many isolates of H. pylori were determined by the air-dried microplate method, and the results indicated that it could be favourably applied to susceptibility testing of H. pylori isolates. The air-dried microplate method has important advantages over the agar plate dilution method in that the handling time of H. pylori is much shorter and reliability is enhanced, thus this method will have a significant positive impact on routine microbiological laboratory work.

REFERENCES


