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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 MIC_{50} and MIC_{90} 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.

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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 MIC_{50} values (at which 50 % of the isolates were inhibited), MIC_{90} 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 1×10^7 to 1×10^8 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 5×10^5–5×10^6 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 (O_2, 10 %; CO_2, 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^T 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 MIC_{50} and MIC_{90} 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 MIC_{50} values, MIC_{90} 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 1. Susceptibility of 253 *H. pylori* isolates to clarithromycin and amoxycillin as determined by the air-dried microplate method and reference agar dilution method

Antimicrobial agent	Method	MIC (μg ml^{-1})												MIC_{50}	MIC_{90}	MIC range
		⩽ 0·015	0·03	0·06	0·12	0·25	0·5	1	2	4	8	16	32	> 32			
Clarithromycin	Air-dried microplate	27	65	73	16	4	1	0	0	5	3	14	18	27	0·06	> 32	⩽ 0·015–> 32
	Agar dilution	46	50	49	30	10	1	0	1	4	8	15	15	24	0·06	32	⩽ 0·015–> 32
Amoxycillin	Air-dried microplate	150	52	25	13	7	4	2	0	0	0	0	0	0	⩽ 0·015	0·12	⩽ 0·015–1
	Agar dilution	165	42	26	6	8	4	2	0	0	0	0	0	0	⩽ 0·015	0·06	⩽ 0·015–1

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

A value of 0 indicates that the two methods yielded the same MICs. A value of +1 indicates that MICs determined by the air-dried microplate method were one twofold dilution higher than determined by the agar dilution method, etc.

Antimicrobial agent	Air-dried microplate MIC/agar dilution MIC (log_2)						
	−3	−2	−1	0	+1	+2	+3
Clarithromycin	1	7	60	114	54	16	1
Amoxycillin	0	5	11	199	31	6	1

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 43504^T, 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 CO_2 concentration. The MIC values of macrolide antibiotics for *H. pylori* isolates in particular are significantly affected by the presence of CO_2 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 CO_2 atmosphere (Kobayashi *et al.*, 1997) and an AnaeroPack method that generates a low CO_2 atmosphere (Kobayashi *et al.*, 2001). In the guidelines established by the NCCLS (1999), the MIC interpretive standard of clarithromycin for *H. pylori* is defined as: susceptible, ⩽ 0·25 μg ml^{-1}; intermediately resistant, 0·5 μg ml^{-1}; resistant, ⩾ 1 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.

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