A new semi-solid agar dilution method for determining amoxycillin, clarithromycin and azithromycin MICs for *Helicobacter pylori* isolates


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The MICs of amoxycillin, clarithromycin and azithromycin for 26 strains of *Helicobacter pylori* were determined using Mueller–Hinton semi-solid agar (SSA) without CO₂ incubation, as in the conventional agar dilution method. The tested *H. pylori* strains grew satisfactorily in the SSA method within 48 h of incubation. Reasonable values of MICs of three antibiotics for these strains were obtained by this method, avoiding the inactivation of macrolides due to acidification of the medium. By this method, the MICs of the antibiotics for the reference strains were within the acceptable NCCLS ranges for quality control.

**Introduction**

Since *Helicobacter pylori* was first isolated from the gastrointestinal mucosa of patients with peptic ulcer disease in 1983, it has often been implicated in chronic gastritis and peptic ulcer disease. Attention has focused on the antimicrobial chemotherapy needed to eradicate the bacteria located in the gastric mucosa of patients. Currently, some treatment regimens with antibiotics, especially macrolides and amoxycillin, have been successfully used to eradicate bacteria and to reduce inflammation of the gastric mucosa.

*H. pylori* is a fastidious, slow-growing bacterium that take 3–4 days at 35°C to form visible colonies in rich culture medium under microaerophilic conditions. During the long incubation in CO₂ needed to determine the MIC of antibiotics by conventional agar dilution methods, macrolide antibiotics in the test medium may be inactivated by acidification of the medium. MICs are markedly greater than those determined in medium adjusted to neutral pH. We found a method of determining the MICs for *H. pylori* strains, using a semi-solid agar (SSA) medium under aerobic conditions, and confirmed that the method was suitable for determining the MICs of various types of antibiotics including macrolides.

In the present study, the activities of clarithromycin, azithromycin and amoxycillin against 26 strains of *H. pylori* were determined by the SSA dilution method under aerobic conditions and compared with those determined by a conventional agar dilution method in a 10% CO₂ atmosphere.

**Materials and methods**

**Test strains**

Twenty-six strains of *H. pylori*, used in this study, were freshly isolated from gastric biopsy specimens of patients. The specimens were placed in a Seed Tube ‘HP’ (Eiken Chemical Co. Ltd, Tokyo, Japan), transported to the microbiology laboratory within 6 h at 4°C, homogenized with sterile physiological saline and then cultured on both selective and non-selective agar media. The former contained lysed horse blood agar with vancomycin, polymyxin B, trimethoprim and amphotericin B and the latter was Columbia agar with 5% sheep blood (Becton Dickinson, Cockeysville, MD, USA). The agar plates were incubated for 7 days at 35°C in an incubator (Tabai Espec Co. Ltd, Kanagawa, Japan) in an atmosphere containing 10% CO₂. *H. pylori* was identified by its requirement for a 10% CO₂ atmosphere for growth and by its typical morphology and positive biochemical tests (oxidase, catalase and urease).

**Antimicrobial agents**

Clarithromycin (Taisho Pharmaceutical Co. Ltd, Tokyo, Japan), azithromycin (Pfizer Pharmaceutical Co. Ltd, Tokyo, Japan) and amoxycillin (Fujisawa Pharmaceutical Co. Ltd, Osaka, Japan) were used as sterile powders of known potency.

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Susceptibility testing

MICs were determined by the agar dilution method with brucella agar (Difco Laboratories, Detroit, MI, USA), supplemented with 5% horse blood and containing antibiotics at concentrations of 0.002–128 mg/L. Five microlitres of each bacterial suspension (10^6 cfu/mL) was inoculated on to the surface of agar plates. The agar plates were incubated at 35°C for 72 h under a 10% CO₂ atmosphere in a CO₂ incubator (Tabai Espec Co. Ltd). The MIC was defined as the lowest concentration of the antibiotic which completely inhibited visible bacterial growth.

The SSA method was used with Muller–Hinton broth (Difco) supplemented with 10% horse serum and 0.2% Bacto Agar (Difco) in each well of 96-well microplates (eight-well strips; Evergreen Scientific, Los Angeles, CA, USA), containing a test antibiotic in concentrations of 0.002–128 mg/L. The test media were inoculated with the bacterial suspension to make 10^7 cfu/mL and incubated aerobically at 35°C for 48 h; the MIC was then defined as the lowest concentration of a test antibiotic which completely inhibited visible bacterial growth in the upper layer of cultured medium in the wells (Figure). Staphylococcus aureus ATCC 29213 and Escherichia coli ATCC 25922 were used as reference strains for quality control.

Results and discussion

By the SSA method, the MICs of amoxycillin for the 26 strains tested ranged from 0.004 to 0.06 mg/L, which were somewhat lower than those (0.008–0.25 mg/L) determined by the agar dilution method (Table). No strains resistant to amoxycillin were found in H. pylori strains tested by either method. By the SSA method, the clarithromycin MIC was 0.004–0.06 mg/L for 22 of the 26 strains and from 2 to 32 mg/L for the other four strains. By the agar dilution method, clarithromycin MICs ranged from 0.016 to 0.5 mg/L for 22 of the 26 strains, while the other four strains were in the resistant range (MICs = 32–128 mg/L). Furthermore, a marked difference was found between the azithromycin MICs determined by the two methods; the azithromycin MICs determined by the agar dilution method were markedly shifted towards the resistant range, as compared with those determined by the SSA method.

The discrepancy in the MICs of clarithromycin and azithromycin for H. pylori strains determined by the two
A new method of determining MIC for *H. pylori*

| Antibiotic | Species | Method       | 0.002 | 0.004 | 0.008 | 0.016 | 0.03  | 0.06  | 0.12  | 0.25  | 0.5   | 1     | 2     | 4     | 8     | 16    | 32    | 64    | 128   | >128  |
|------------|---------|--------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Amoxicillin | *H. pylori* | SSA agar dilution | 8   | 10   | 5    | 1    | 2    |      |       |       |       |       |       |       |       |       |       |       |       |       |
|            | *S. aureus* | SSA agar dilution |    |       |      |      |      | 1    |       |       |       |       |       |       |       |       |       |       |       |       |
|            | *E. coli* | SSA agar dilution |    |       |      |      |      |       |       |       |       |       |       |       |       |       |       |       |       |       |
| Clarithromycin | *H. pylori* | SSA agar dilution | 2   | 6    | 11   | 1    | 2    | 1    | 2     | 1     |       |       |       |       |       |       |       |       |       |       |
|            | *S. aureus* | SSA agar dilution |    | 1    | 1    | 1    | 13   | 5    | 1     |       |       |       | 2     | 1     | 1     |       |       |       |       |       |
|            | *E. coli* | SSA agar dilution |    |       |      |      |      |      |       |       |       |       |       |       |       |       |       |       |       |       |
| Azithromycin | *H. pylori* | SSA agar dilution | 3   | 2    | 11   | 4    | 1    | 1    | 1     | 1     |       |       |       |       | 2     |       |       |       |       |       |
|            | *S. aureus* | SSA agar dilution |    | 1    | 9    | 8    | 4    | 1     | 1     |       |       |       |       |       | 4     |       |       |       |       |       |
|            | *E. coli* | SSA agar dilution |    |       |      |      |      |      |       |       |       |       |       |       |       |       |       |       |       |       |

*S. aureus* ATCC 29213.

*E. coli* ATCC 29222.
methods, was considered to result from inactivation of macrolide antibiotics in the agar dilution method as a result of acidification of the test medium (due to \( \text{CO}_2 \)). The present results concerning the inactivation of macrolides coincide with recently published data.\(^6,7\) MICs of macrolides for \( \text{H. pylori} \) strains were determined by a conventional agar dilution method with Muller–Hinton agar, supplemented with 5\% horse blood at different \( \text{pH} \) values (\( \text{pH} \) 6.0, 7.0 and 8.0), under 10\% CO\(_2\) atmosphere; the MICs obtained with the medium adjusted to \( \text{pH} \) 6.0 were greater than those obtained with media adjusted to \( \text{pH} \) 7.0 or \( \text{pH} \) 8.0. Although acidification of the medium could be inhibited by adding 0.1 M phosphate buffer (a neutral \( \text{pH} \)), inhibition of \( \text{H. pylori} \) growth in the test medium was unavoidable. When the MICs of macrolide antibiotics were determined under 10\% \( \text{CO}_2 \) atmosphere by the SSA method, the MICs were higher (data not shown).

To confirm the reliability of the MICs determined by the SSA method, a quality control experiment was performed according to published guidelines.\(^8\) The MICs of amoxy-clin, clarithromycin, and azithromycin for two reference strains, \( \text{S. aureus A TCC 29213} \) and \( \text{E. coli A TCC 25922} \), were within the acceptable ranges for the quality control of NCCLS.\(^8\) However, MICs of clarithromycin and azithromycin, as determined by the agar dilution method, for \( \text{S. aureus A TCC 29213} \) were beyond the acceptable range, indicating inactivation of the antibiotics as a result of \( \text{CO}_2 \) incubation.

All of the tested strains of \( \text{H. pylori} \) grew satisfactorily in the aerobic conditions used for the SSA method within 48 h incubation. We concluded that the SSA method was reliable for assessing the susceptibility of \( \text{H. pylori} \) strains to various antibiotics including macrolides.

### References


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