Antibiotic MICs and short time–killing against *Helicobacter pylori*: therapeutic potential of kanamycin

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We compared the susceptibility of *Helicobacter pylori* to several antibiotics, expressed as MICs and as bactericidal effectiveness in short (3 h) time–killing studies. Of the antimicrobial agent tests, clarithromycin and amoxycillin had the lowest MIC50, 0.063 and 0.125 mg/L respectively, for 24 strains of *H. pylori*. Minocycline, levofloxacin and lansoprazole followed, with MIC50s of 0.5, 1, and 2 mg/L, respectively. Three-hour time–killing studies using a standard strain demonstrated a different pattern. At 4 x MIC, kanamycin, metronidazole and clarithromycin produced 4.4, 2.6 and 2.1 log decreases in viability, whereas the remaining seven antibiotics (including amoxycillin) were less bactericidal. Amoxycillin’s lack of bactercidal activity during brief incubations was confirmed by examining several different clinically isolated *H. pylori* strains. Clarithromycin’s effect, on the other hand, was strain- and concentration-dependent. Kanamycin was the most potent antibiotic in short time–killing studies, with concentrations of 1 x MIC and 4 x MIC producing a reduction of more than 2 and 4 log respectively in all ten strains. Our data suggest that the MIC of antimicrobial agents against *H. pylori* does not necessarily predict their activity in short time–killing studies. Furthermore, our short time–kill data suggest kanamycin as a potential therapeutic choice for *H. pylori* infection, even though this agent’s MIC would suggest limited activity.

**Introduction**

The association of *Helicobacter pylori* with active gastritis and gastroduodenal ulcer has been demonstrated by many investigators. In patients with mucosal *H. pylori* infection, eradication of this microorganism seems to cure both the infection and the accompanying ulcer disease. However, eradication of *H. pylori* is extremely difficult in vivo, even though this organism appears sensitive to many antibiotics in vitro. In-vitro susceptibility testing of *H. pylori* is difficult because this microorganism grows slowly and is fastidious. Moreover, testing methods are controversial because the results do not always correlate with the clinical outcome. For example, in-vitro susceptibility testing indicates that amoxycillin is one of the more active agents against *H. pylori*, and has led to extensive study of this agent for therapy of such infections. However, amoxycillin produces long-term *H. pylori* eradication in only 20% of patients. Such observations clearly show that traditional MIC determinations are inadequate for determining an antibiotic’s clinical effectiveness against this mucosa-associated bacterium. The high rate of clinically observed relapse, and the possible existence of a subpopulation of cells that are not actively replicating, suggest that bactercidal therapy may be required in order to eradicate *H. pylori*. This concept has led several investigators to use non-conventional methods for evaluating antibiotics’ bactercidal activity against this organism, but the optimal therapeutic regimen has not yet been identified.

**Materials and methods**

**Organisms tested**

Twenty-three strains of *H. pylori* were isolated from patients with gastritis, gastric ulcer and/or duodenal ulcer...
at Toho University School of Medicine (Tokyo, Japan). Strain CPY 2052 was kindly given by Professor Okita (Yamaguchi University School of Medicine, Yamaguchi, Japan). Isolates were frozen at -80°C in Brucella broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 10% fetal bovine serum (Immuno Biological Laboratories, Fujioka, Japan) and 10% DMSO (Wako Pure Chemical Industries, Ltd, O saka, Japan) until used for MIC determination and bactericidal studies.

Antimicrobial agents

A moxycillin, clarithromycin, kanamycin, metronidazole and lansoprazole were obtained from Fujisawa Pharmaceutical Co., Ltd (Osaka, Japan), Dainippon Pharmaceutical Co., Ltd (Tokyo, Japan), Shionogi & Co., Ltd (Osaka, Japan), Takeda Chemical Industries, Ltd (Osaka, Japan) and Takeda Chemical Industries, Ltd (Tokyo, Japan) respectively. Cefacrol, clindamycin, fosfomycin, levofloxacin and minomycin were obtained from Shionogi & Co., Ltd (Osaka, Japan), Japan Upjohn Co., Ltd (Tokyo, Japan), Meiji Seika Ltd (Tokyo, Japan), Daiichi Pharmaceutical Co., Ltd (Tokyo, Japan) and Lederle Japan, Ltd (Tokyo, Japan) respectively.

Antimicrobial susceptibility tests

The MICs of antibiotics were determined by agar dilution method using Brucella agar (BBL) supplemented with 2% soluble starch and 3% horse serum (Nippon Bio-Test Laboratories, Tokyo, Japan), in which antibiotics were serially diluted in a two-fold series at concentrations between 0.063 and 128 mg/L. Clarithromycin, metronidazole and lansoprazole were initially dissolved in methanol and were then further diluted with sterile water. Isolates were grown for 24 h in Brucella broth with 10% fetal bovine serum to provide a turbidity of approximately 10^9 cfu/mL. Bacterial suspensions were diluted 1:100 with the same medium. A 0.01 mL sample of these suspensions was inoculated on to the agar using a micro-planter (Sakuma Industry, Tokyo, Japan). The plates were incubated for 5 days at 37°C in a humid, microaerophilic environment (Anero pack Campylo, Mitsubishi Gas Kagaku Co., Ltd, Tokyo, Japan). The MIC was defined as the lowest concentration that inhibited visible growth of bacteria, disregarding any barely visible haze.

Short-term bactericidal studies

Bactericidal studies were performed in Brucella broth containing 10% fetal bovine serum incubated in a humid, microaerophilic environment. Antimicrobics at concentrations of 0, 1, 4, 16 or 64 x MIC of each strain were prepared in broth medium, and bacteria were incuobated to a final concentration of 10^7 cfu/mL. Samples (0.1 mL) were removed after 0 and 3 h incubation, and were serially diluted ten-fold. They were then spread on to blood agar (Blood agar base no. 2, Oxoid, Basingstoke, UK) supplemented with defibrinated horse blood (Nippon Bio-Test Laboratories) and 2% soluble starch. After 5 days of incubation under microaerophilic conditions at 37°C, the number of colonies was determined.

Results

Antibiotic susceptibility

The Table shows the MICs obtained for 24 strains of H. pylori. Clarithromycin and amoxycillin were the most active antibiotics tested, with MIC_{90}s of 0.063 and 0.125 mg/L respectively. They were followed in order by minocycline, levofloxacin and lansoprazole, with MIC_{90}s of 0.5, 1 and 2 mg/L respectively. The MICs reported here are consistent with those in the previous literature.

Comparative bactericidal activity against H. pylori

Changes of viability after 3 h incubation with 1, 4, 16 and 64 x MIC of ten antimicrobial agents were examined using strain CPY 2052 (Figures 1 and 2). At 4 x MIC, kanamycin, metronidazole and clarithromycin produced viability decreases of 4.4, 2.6, and 2.1 log respectively; higher concentrations were more bactericidal against this strain. The remaining seven antibiotics (including amoxycillin) were less bactericidal, producing viability decreases of at most 1.0 log at 4 x MIC.

Short time–killing study of various antibiotics against H. pylori clinical isolates

Amoxycillin. Viability changes after 3 h incubation with 1, 4, 16 and 64 x MIC of amoxycillin were examined for eight clinical isolates of H. pylori (Figure 3). Although a

| Table. MICs of various antibiotics against H. pylori (24 strains) |
|---------------------------------|----------------|----------------|
| **Antibiotics** | range | MIC (mg/L) |
| A moxycillin | 0.032–64 | 0.125 | 0.25 |
| Clarithromycin | 0.0176–0.5 | 0.063 | 0.25 |
| Kanamycin | 0.5–64 | 8 | 16 |
| Metronidazole | 1–32 | 8 | 16 |
| Lansoprazole | 1–4 | 2 | 2 |
| Cefacrol | 0.25–128 | 8 | 128 |
| Clindamycin | 1–128 | 4 | 16 |
| Fosfomycin | 2–64 | 32 | 64 |
| Levofloxacin | 0.25–64 | 1 | 32 |
| Minocycline | 0.125–2 | 0.5 | 2 |
**MICs and short time–killing against H. pylori**

**Figure 1.** Short time–killing activity of various antibiotics against H. pylori CPY2052: (a) amoxycillin; (b) clarithromycin; (c) kanamycin; (d) metronidazole; (e) lansoprazole. ○, Control; △, 1 × MIC; □, 4 × MIC; ●, 16 × MIC; ▲, 64 × MIC.

**Figure 2.** Short time–killing activity of various antibiotics against H. pylori CPY2052; (a) cefacrol; (b) clindamycin; (c) fosfomycin; (d) levofloxacin; (e) minocycline. Symbols as in Figure 1.

**Figure 3.** Short time–killing activity of amoxycillin against eight clinical isolates of H. pylori: (a) control; (b) 1 × MIC; (c) 4 × MIC; (d) 16 × MIC; (e) 64 × MIC.
concentration of $4 \times$ MIC of amoxycillin for each strain slightly decreased bacterial numbers in three of the eight strains tested, there was no apparent enhancement of bactericidal activity at higher concentrations. Thus, amoxycillin appears to exhibit little bactericidal activity against H. pylori after short-term incubation.

Clarithromycin and kanamycin. Changes in viability after 3 h incubation with 1, 4, 16 and $64 \times$ MIC of clarithromycin and kanamycin for each strain were examined for ten clinical isolates of H. pylori (Figures 4 and 5). A viability decrease of $>1$ log was observed in two strains incubated with $1 \times$ MIC and seven strains incubated with $4 \times$ MIC of clarithromycin. At $64 \times$ MIC, clarithromycin induced a viability reduction of $>3$ log in all strains tested. Interestingly, kanamycin at $1 \times$ MIC produced a viability decrease of $>2$ log against all clinically isolated strains. This bactericidal activity is considerably more potent than that of any other antibiotic at an equivalent concentration; indeed, higher concentrations of kanamycin induced decreases of more than 4 and 5 log against all strains at $4 \times$ and $16 \times$ MIC respectively.

Kanamycin/clarithromycin and kanamycin/amoxycillin combinations. A short (3 h) time–killing study was performed using $1 \times$ MIC of amoxycillin and clarithromycin, either alone or in combination with $1 \times$ MIC of kanamycin. No synergy was observed in these studies.

Discussion
This study demonstrates that an antibiotic’s MIC for H. pylori does not necessarily parallel its activity in short time–killing studies, and that kanamycin may have a therapeutic role in the treatment of H. pylori infection.

Numerous clinical trials of various treatments for
H. pylori infection have shown that there are many antimicrobial agents which appear excellent in vitro but are completely ineffective in vivo.\textsuperscript{10-12} These observations raise two important questions: first, whether the usual method for antimicrobial susceptibility testing accurately reflects interactions between the organism and antimicrobial agents at the site of infection, and second, which tests are most appropriate for estimating the clinical efficacy of antibiotics.

Since H. pylori resides in a protected niche and/or the gastric mucosa, where host antibacterial defences have limited effectiveness, one might expect that an effective antimicrobial drug must be bactericidal rather than bacteriostatic. Time–kill curves of H. pylori exposed to several drug concentrations measure bactericidal activity over time, and may reveal differences in activity between agents with similar MICs.

Amoxycillin is a generally well-tolerated antibiotic with good activity against H. pylori in vivo. It has been used in many clinical trials aimed at eradicating this bacterium from the gastric mucosa.\textsuperscript{18,19} Results of these trials, however, have been disappointing due to rapid bacterial recolonization.\textsuperscript{13} Similar results have been reported by Karita et al.\textsuperscript{20} in the nude mouse model of H. pylori infection: four weeks after the end of treatment with amoxycillin, the number of colonies recovered was not significantly lower than in the control group.

Our results indicate that amoxycillin has little bactericidal activity in 3 h incubations in vitro; no concentration-dependent killing was observed against any strain tested. Several other investigators have also reported that amoxycillin lacks bactericidal activity, especially over short incubation periods. For example, Berry et al.\textsuperscript{21} have reported that viability of H. pylori NCTC 11637 did not decrease following incubation for 3 h with amoxycillin concentrations of 10 and 100 \times \text{MIC}, although they found evidence for a bactericidal effect when the drug concentration was increased to 1000 \times \text{MIC}. Similarly, Midolo et al.\textsuperscript{22} have reported that amoxycillin time–killing curves do not show concentration dependence against strain NCTC 11637. Our data are largely consistent with these two earlier reports, and further expanded amoxycillin’s demonstrated lack of short-term bactericidal activity to several clinical isolates.

Poor penetration into gastric mucosa and the drug’s pharmacokinetics in the stomach are factors that may possibly contribute to amoxycillin’s limited clinical efficacy. Only a few studies have determined the concentration of amoxycillin present in the gastric mucosa. It has been shown that 1 g of amoxycillin orally produced a concentration of \textgt0.1 mg/g in the mucosa after 30 min, but concentrations had fallen below this level by 60 and 90 min.\textsuperscript{23} In a similar study, McNulty et al.\textsuperscript{24} found concentrations ranging from 0.01 to 0.32 mg/g at 90 min after administration of a 500 mg oral dose. These observations led us to examine a maximum amoxycillin concentration of 64 \times \text{MIC} using a 3 h incubation time. We believe that such experiments may reflect the in-vivo activity of amoxycillin better than the MIC does. Further studies considering pharmacokinetic parameters of amoxycillin in the stomach may be necessary to define exactly the efficacy of this antibiotic against H. pylori infection.

Of the ten antimicrobial agents tested, MIC\textsubscript{50}s suggested that clarithromycin, amoxycillin and minocycline were the most active; these were followed in order by levofloxacin, lansoprazole and clindamycin. In contrast, only three antibiotics (kanamycin, clarithromycin and metronidazole) produced concentration-dependent killing against strain CPY 2052 in short time–killing studies; no such effect was observed with amoxycillin, lansoprazole, cefacrol, clindamycin, fosfomycin, levofloxacin or minocycline. Interestingly, the most potent activity in short-time–killing studies was shown by kanamycin, with 1 \times \text{MIC} of this antibiotic producing a viability decrease of \textgt2 log in all ten strains tested.

In any type of bacterial infection, the first step in the search for a suitable therapeutic agent is the evaluation of the causative organism’s susceptibility in vitro to potentially useful agents. Drugs that prove less active in vitro are unlikely to be readily considered for therapy. In reviewing the MIC ranges of more than 60 antimicrobial agents against H. pylori, Glupczynski\textsuperscript{17} found that kanamycin (MIC = 1–4 mg/L) was clearly less active than most of the remaining agents. Our data also showed that kanamycin was less active against clinical isolates, judging from its MIC\textsubscript{50} (8 mg/L). On the other hand, kanamycin at 1 \times \text{MIC} produced a viability decrease of \textgt2 log against all clinical isolates. It appears likely that kanamycin concentrations of 1 \times \text{MIC} may be achievable at the site of H. pylori infection because 1 g or more dose of kanamycin can be administered orally. Thus, our data suggest the necessity for re-evaluating the in-vitro and in-vivo efficacy of aminoglycoside antibiotics against H. pylori. Different pH between in-vitro and in-vivo conditions, in addition to the pharmacokinetics of each antibiotic in the stomach, may be possible factors to explain the discrepancy of efficacy of antibiotics.\textsuperscript{25} Unfortunately, the clinical efficacy of kanamycin in eradicating this organism has not yet been investigated.

In conclusion, our observations suggest that susceptibility testing alone may not be sufficient to provide evidence of the clinical potential of anti-H. pylori agents, and that short time–killing studies may be a useful method for evaluating the efficacy of antibiotics. Our results also clearly indicate the potential of kanamycin as a therapeutic strategy against H. pylori infection, despite this agent’s relatively high MIC.

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