Can Aminoglycosides Be Used as a New Treatment for Helicobacter pylori? In vitro Activity of Recently Isolated isolated Helicobacter pylori

Kyoung Hwa Lee 1, Soon Young Park 1, Su Jin Jeong 1, Da Hyun Jung 1, Jie-Hyun Kim 1, Seok Hoon Jeong 1, Il-Mo Kang 4,*, and Young Goo Song 1,*

1 Division of Infectious Diseases, Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Korea.
2 Division of Gastroenterology, Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Korea.
3 Department of Laboratory Medicine, Yonsei University College of Medicine, Seoul, Korea.
4 Korea Institute of Geoscience and Mineral Resources, Daejeon, Korea

ABSTRACT

Background: Smectite can serve as a drug delivery system and gentamicin-intercalated smectite hybrids are expected to supersede the standard therapy for Helicobacter pylori eradication. The aim of this study was to confirm whether the minimum inhibitory concentration (MIC) of aminoglycosides applied as smectite hybrids remained low against recently isolated H. pylori strains.

Materials and Methods: A total of 140 strains were collected for a minimum period of 3 years. Antimicrobial susceptibility tests were performed, and the MICs of eight antibiotics (amoxicillin, clarithromycin, metronidazole, tetracycline, levofloxacin, gentamicin, netilmicin, and tobramycin) were determined by using the Epsilometer test and following the European Committee on Antimicrobial Susceptibility Testing recommendations.

Results: The resistance rate of clarithromycin was high, up to 30.7%, although it is a major antimicrobial agent used in standard therapy. The MIC50 and MIC90 of gentamicin (0.25 mg/L and 0.75 mg/L) and netilmicin (0.19 mg/L and 0.75 mg/L) were lower than other alternative therapies for H. pylori eradication. In clarithromycin-resistant strains, the MIC50 was 0.25 mg/L and the MIC90 was 1 mg/L for gentamicin; for netilmicin, the values were 0.25 mg/L and 0.75 mg/L, respectively.

Conclusion: Through the use of gentamicin and netilmicin, which have low MICs for H. pylori, aminoglycoside-intercalated smectite hybrids are expected to emerge as a new standard therapy for H. pylori eradication.

Keywords: Helicobacter pylori; Clarithromycin; Aminoglycosides

INTRODUCTION

The global prevalence of Helicobacter pylori infection remains high; there were approximately 4.4 billion individuals with H. pylori infection worldwide in 2015, although the infection prevalence varies by country. In South Korea, one of every two healthy people is a carrier of H. pylori [1-3]. This gram-negative bacillus is associated with peptic ulcers, mucosa-associated
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**ORCID IDs**
- Kyoung Hwa Lee  
  https://orcid.org/0000-0003-0033-1398
- Il-Mo Kang  
  https://orcid.org/0000-0002-0255-379X
- Young Goo Song  
  https://orcid.org/0000-0002-0733-4156

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**Conflict of Interest**
No conflicts of interest.

**Author Contributions**
- Conceptualization: YGS.
- Data curation: SYP.
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Lymphoid tissue lymphoma, and gastric cancer. Therefore, the eradication of *H. pylori* is a critical aspect of disease management and prevention [4, 5]. However, the eradication rate of *H. pylori* as a standard therapy based on amoxicillin and clarithromycin, exhibits a decreasing trend, in the range of 74.6%–75.8% in South Korea, which represents a high burden for the country of *H. pylori* [6, 7]. This failure of *H. pylori* eradication results from antimicrobial resistance, especially to clarithromycin [8-11]. Alternative approaches have been explored, such as sequential therapy, concomitant therapy, quinolone- or rifabutin-containing therapy, and a tailored therapy based on antimicrobial susceptibility have been introduced; however, there is still controversy in the regimen change and these do not provide a satisfactory substitute to the existing standard therapy [11-19]. Thus, a novel and efficient *H. pylori* eradication regimen should be developed.

Aminoglycosides not previously been considered for *H. pylori* eradication, even though they have been conventionally used for aerobic Gram-negative bacterial infections. Aminoglycosides cannot be absorbed by the gastrointestinal tract owing to their polar, water-soluble nature; they have very poor intestinal membrane permeability [20]. Thus, they are usually delivered through intravenous or intramuscular forms, which are not practical for *H. pylori* eradication. In contrast, because *H. pylori* adheres to the gastric epithelium and lives in the gastric mucosa layer [21], if aminoglycosides are applied as coating agents to the gastric wall, their poor absorption characteristics can prove to be an advantage for local therapy.

Therefore, in the previous study, we synthesised gentamicin-intercalated smectite hybrid (S-GEN) complexes as a novel therapeutic agent. In a murine model, S-GEN released gentamicin to the gastric wall stably and the therapeutic effect was not inferior to the conventional standard therapy [22]. Although aminoglycosides have been used for decades, few studies have determined the MIC values of aminoglycosides against *H. pylori* over the last two decades [23, 24]. Therefore, for the clinical use of S-GEN, it is necessary to confirm the MIC of aminoglycosides against recently isolated *H. pylori*.

The aim of this study was to confirm whether the MIC of aminoglycosides remained low against recently isolated *H. pylori* strains. If the MIC remained sufficiently low, the results may indicate the possibility for the development of new aminoglycosides-based therapeutic agents against *H. pylori* through using a smectite hybrid complex.

**MATERIALS AND METHODS**

1. *Helicobacter pylori* strains in the study

We collected 222 strains of *H. pylori* which were isolated from 1,422 patients, at Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul, Korea, between March 2015 and February 2018. If *H. pylori* infection was suspected during endoscopy, a gastric tissue biopsy and *H. pylori* culture were routinely performed by gastroenterologists and physicians of laboratory medicine in our hospital. We used only collected *H. pylori* strains without any patient-identifying information, and institutional review board approval was waived because the research did not involve human subjects. The isolated strains were subcultured with eight antimicrobials (amoxicillin, clarithromycin, metronidazole, tetracycline, levofloxacin, gentamicin, netilmicin, and tobramycin) and susceptibility testing was conducted. Among 222 *H. pylori* strains, 37 strains did not grow at all in the subculture tests and 45 strains were excluded because some of the susceptibility tests were not reported. As the results of 82
strains could produce confounding factors in the data analysis, we excluded them all. Finally, 140 *H. pylori* strains, for which the results of the susceptibility subculture tests against eight antimicrobial agents were analyzed (Supplementary Table 1).

2. **Isolation of Helicobacter pylori**

*H. pylori* strains were cultured on egg yolk emulsion (EYE) agar plates (Yuhan LabTech, Seoul, Korea) and their growth was closely observed. The EYE agar contained 43.82 µg/mL Columbia agar, 112.36 µL/mL EYE, 11.23 µL/mL IsoVitaleX, and 45.0 µg/mL 2,3,5-triphenyltetrazolium chloride for colony staining [25]. The plates were stored in a multi-gas incubator (microaerophilic atmosphere: 10% CO₂, 5% O₂, and 85% N₂) at 37°C for 3–7 days.

The isolation of *H. pylori* was performed on the basis of colony morphology and was confirmed via matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF) using a Microflex LT system (Bruker Daltonics, Bremen, Germany). The measured profiles were compared with a database by using MALDI Biotyper 3.1 software (Bruker Daltonics, Bremen, Germany).

3. **Antimicrobial susceptibility test**

The *in vitro* MICs of five kinds of antibiotics: amoxicillin, clarithromycin, metronidazole, tetracycline, levofloxacain and three kinds of aminoglycosides: gentamicin, netilmicin and tobramycin against recent clinical isolates of *H. pylori* were tested. The MICs of these eight antibiotics against *H. pylori* were determined via the Epsilometer test (E-test) using an E-strip (BioMerieux SA, France) three times. E-test is reliable and shows excellent agreement with agar dilution and broth microdilution tests [26]. The MICs were determined in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [27]. Their clinical breakpoints for amoxicillin, clarithromycin, metronidazole, tetracycline, and levofloxacin are >0.125 mg/L, >0.5 mg/L, >8 mg/L, >1 mg/L, and >1 mg/L, respectively. Because breakpoints for aminoglycosides against *H. pylori* have not been provided, we compared the MIC₅₀, the MIC₉₀, and the MIC range. We performed quality control by using a standard strain, *H. pylori* ATCC 43504 (NCTC 11637, Manassas, VA, USA), with every batch.

4. **Statistical analysis**

Categorical variables were described as frequencies and percentages (%). McNemar’s test was used to analyze the association of two different categorical variables. The resistance rates of two different antibiotic groups were compared. All *P*-values were two-tailed, and values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS Version 23 software (IBM Corp., Armonk, NY, USA).

**RESULTS**

1. **Antimicrobial susceptibility of eight antimicrobial agents against Helicobacter pylori**

Based on the results of the antimicrobial susceptibility test, twelve strains (8.6%) were resistant to amoxicillin when the breakpoint of 0.125 mg/L was applied according to the EUCAST recommendation. The resistance rate of clarithromycin, which is one of the standard treatment methods for *H. pylori* eradication, was 30.7%. When the first line
standard therapy failed, the recommended second line of treatment was a combination of metronidazole and levofloxacin. Their resistance rates were also high at 26.4% and 37.9%, respectively.

The definition of resistance to aminoglycosides in *H. pylori* organisms has not been established yet. Thus, we cannot distinguish the number of *H. pylori* strains that are resistant to aminoglycosides. The MIC$_{50}$ and MIC$_{90}$ of gentamicin and netilmicin were 0.19-0.25 mg/L and 0.75 mg/L, respectively (Table 1). And the number of *H. pylori* isolates in each MIC and total MIC distributions of all 140 strains are shown in Figure 1 for each of the eight antibiotics.

### 2. Clarithromycin-resistant *Helicobacter pylori*

As mentioned previously, the high resistance rate to clarithromycin in South Korea is a major cause of *H. pylori* eradication failure. The recently clinical isolated *H. pylori* strain has a 30% resistance rate of clarithromycin, as shown in this study. Therefore, we analyzed resistance patterns of other antimicrobial agents in clarithromycin-resistant *H. pylori* strains. Among the 43 clarithromycin-resistant strains, eight (18.6%) were resistant to amoxicillin. Metronidazole and levofloxacin showed a higher resistance rate of 34.9% and 48.8%, respectively. The resistance rate of tetracycline was also 18.6%. For the aminoglycosides, the MIC$_{50}$ was 0.25 mg/L and the MIC$_{90}$ was 1 mg/L for gentamicin; for netilmicin, the values were 0.25 mg/L and 0.75 mg/L, respectively. Finally, for tobramycin, these values were 1 mg/L and 2 mg/L, respectively (Table 2).

### 3. Susceptibility of aminoglycosides and cumulative percentage

The breakpoint for *H. pylori* in aminoglycosides has not been studied since it has not attempted to use aminoglycosides as *H. pylori* therapy. Therefore, we conservatively estimated the break point at 1 mg/L and compared the results with the other five antibiotics. The resistance rate was 3.6% and 2.1% for gentamicin and netilmicin, respectively, and 40.0% for tobramycin. In the 43 clarithromycin-resistant strains, the MICs of gentamicin and netilmicin were still low, with a 7% resistance rate based on a resistance breakpoint of >1 mg/L (Table 3). The dotted line in Figure 1 represented the breakpoint according to the EUCAST recommendations for each antibiotic, and was also indicated as 1 mg/L for aminoglycosides antibiotics. The strain on the left side of the dotted line is susceptible strains to the corresponding antibiotic and the strain on the right from the break point dotted line is resistant to the corresponding antibiotic.

Dividing the number of all strains below the specific MIC by the total number of *H. pylori* strains (the number of strains below specific MIC/140 *100) means cumulative susceptibility percentage in the corresponding MIC. The cross point with the break point line and

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**Table 1. Resistance rate, MIC$_{50}$ and MIC$_{90}$ of antibiotics against *Helicobacter pylori* strains (n = 140)**

| Antibiotics  | Resistance rate, n (%) | MIC$_{50}$ (mg/L) | MIC$_{90}$ (mg/L) | MIC range (mg/L) |
|--------------|------------------------|-------------------|-------------------|------------------|
| Amoxicillin  | 12 (8.6)               | 0.023             | 0.125             | 0.016–32         |
| Clarithromycin | 43 (30.7)            | 0.064             | 256               | 0.016–256        |
| Metronidazole | 37 (26.4)            | 0.5               | 256               | 0.016–256        |
| Tetracycline  | 13 (9.2)               | 0.38              | 1                 | 0.016–6          |
| Levofloxacin  | 53 (37.9)             | 0.5               | 32                | 0.002–32         |
| Gentamicin    | No standard            | 0.25              | 0.75              | 0.016–6          |
| Netilmicin    | No standard            | 0.19              | 0.75              | 0.016–4          |
| Tobramycin    | No standard            | 1                 | 2                 | 0.016–8          |

MIC, minimum inhibitory concentration; MIC$_{50}$, minimum concentration able to inhibit the growth of 50% of organisms; MIC$_{90}$, minimum concentration able to inhibit the growth of 90% of organisms.
Aminoglycosides activity against *H. pylori*

**Figure 1.** Minimum inhibitory concentration distributions of various antibiotics against *Helicobacter pylori* isolates. The dotted line represents the breakpoint in each antibiotic. Resistance break points for MICs against *Helicobacter pylori* were defined based on the recommendations of the European Committee on Antimicrobial Susceptibility Testing. For aminoglycosides, breakpoints for *H. pylori* have not been previously studied; therefore, strict breakpoints >1 mg/L have been applied.

MIC, minimum inhibitory concentration; AMX, amoxicillin; CLR, clarithromycin; MDZ, metronidazole; TET, tetracycline; LVX, levofloxacin; GEN, gentamicin; NET, netilmicin; TOB, tobramycin.

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Aminoglycosides activity against *H. pylori*

Figure 1. (Continued) Minimum inhibitory concentration distributions of various antibiotics against *Helicobacter pylori* isolates. The dotted line represents the breakpoint in each antibiotic. Resistance break points for MICs against *Helicobacter pylori* were defined based on the recommendations of the European Committee on Antimicrobial Susceptibility Testing. For aminoglycosides, breakpoints for *H. pylori* have not been previously studied; therefore, strict breakpoints >1 mg/L have been applied.

MIC, minimum inhibitory concentration; AMX, amoxicillin; CLR, clarithromycin; MDZ, metronidazole; TET, tetracycline; LVX, levofloxacin; GEN, gentamicin; NET, netilmicin; TOB, tobramycin.
cumulative percentage line is close to 100%, the more likely the antibiotic is effective as a therapeutic agent.

4. Comparison of resistance rate in statistical analysis

We compared the resistance rate of each antibiotic via pairing comparisons using McNemar’s test (Table 4). Comparing the resistance rates of the eight antibiotics, netilmicin was the least resistant and gentamicin was the next, but the difference between the two antibiotics was not significant (2.1% vs. 3.6%, \( P = 0.625 \)). In the case of tobramycin, which is a kind of aminoglycosides, the resistance rate was 40% and different from other aminoglycosides. The difference in resistance rate between them was significant. (netilmicin vs tobramycin, 2.1% vs. 40.0%, \( P < 0.001 \); gentamicin vs tobramycin, 3.6% vs. 40.0%, \( P < 0.001 \)). When the resistance rates of the standard therapies with amoxicillin and clarithromycin were checked, the amoxicillin had significantly higher resistance than that of netilmicin (\( P = 0.012 \)), and the difference between amoxicillin and gentamicin was not significant (\( P = 0.065 \)). Clarithromycin showed significantly higher resistance than netilmicin and gentamicin (\( P < 0.001 \)). The resistance of tetracycline, metronidazole, and levofloxacin used as second-line therapy was significantly higher than that of netilmicin (\( P < 0.001 \), except tetracycline (\( P = 0.057 \)).

Table 2. Resistance rate, MIC\(_{50}\) and MIC\(_{90}\) of antibiotics against clarithromycin-resistant strains of Helicobacter pylori (n = 43)

| Antibiotics  | Resistance rate, n (%) | MIC\(_{50}\) (mg/L) | MIC\(_{90}\) (mg/L) | MIC range (mg/L) |
|-------------|------------------------|---------------------|---------------------|------------------|
| Amoxicillin | 8 (18.6)               | 0.032               | 0.25                | 0.016–32         |
| Metronidazole | 15 (34.9)            | 1                   | 256                 | 0.016–256        |
| Tetracycline | 8 (18.6)               | 0.75                | 2                   | 0.016–4          |
| Levofloxacin | 21 (48.8)             | 1                   | 32                  | 0.002–32         |
| Gentamicin  | No standard            | 0.25                | 1                   | 0.047–6          |
| Netilmicin  | No standard            | 0.25                | 0.75                | 0.016–4          |
| Tobramycin  | No standard            | 1                   | 2                   | 0.016–8          |

MIC, minimum inhibitory concentration; MIC\(_{50}\), minimum concentration able to inhibit the growth of 50% of organisms; MIC\(_{90}\), minimum concentration able to inhibit the growth of 90% of organisms.

Table 3. The resistance rate of aminoglycosides against Helicobacter pylori with a breakpoint of >1 mg/L

| H. pylori strain | Antibiotics | Resistance breakpoint (mg/L) | Resistance rate, n (%) |
|------------------|-------------|------------------------------|------------------------|
| All strains (n = 140) | Gentamicin | >1                           | 5 (3.6)                |
|                  | Netilmicin  | >1                           | 3 (2.1)                |
|                  | Tobramycin  | >1                           | 56 (40.0)              |
| Clarithromycin-resistant strains (n = 43) | Gentamicin | >1                           | 3 (7.0)                |
|                  | Netilmicin  | >1                           | 2 (4.7)                |
|                  | Tobramycin  | >1                           | 19 (44.2)              |

*aThe breakpoints of aminoglycosides against Helicobacter pylori have not been studied before; therefore, we conservatively assumed a strict resistance breakpoint of >1 mg/L for estimation of the resistance rate.

Table 4. Comparison of the resistance rates of various antibiotics against Helicobacter pylori strains

| Antibiotics | Resistance rate (%) | NET | GEN | AMX | TET | MDZ | CLR | LVX | TOB |
|-------------|---------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| NET         | 2.1                 | 0.625 | 0.012 | 0.006 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| GEN         | 3.6                 | 0.625 | 0.057 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| AMX         | 8.6                 | 0.012 | 0.065 | >0.999 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| TET         | 9.3                 | 0.065 | 0.057 | >0.999 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| MDZ         | 26.4                | <0.001 | 0.057 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| CLR         | 30.7                | <0.001 | 0.001 | <0.001 | <0.001 | <0.001 | 0.480 | 0.036 | 0.032 |
| LVX         | 37.9                | <0.001 | <0.001 | <0.001 | <0.001 | 0.036 | 0.220 | 0.810 | -    |
| TOB         | 40.0                | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.32 | 0.124 | 0.810 |

Data represent the \( p \)-value of pairwise comparison by McNemar’s test. The breakpoints of aminoglycosides against Helicobacter pylori have not been studied before; therefore, we conservatively assumed a strict resistance breakpoint of >1 mg/L for estimation of the resistance rate.

NET, netilmicin; GEN, gentamicin; AMX, amoxicillin; TET, tetracycline; MDZ, metronidazole; CLR, clarithromycin; LVX, levofloxacin; TOB, tobramycin.
DISCUSSION

In our study, we reported the MIC and resistance rate of various antibiotics, including aminoglycosides, against recently isolated *H. pylori* strains. As expected, the resistance rate to clarithromycin was high (up to 30.7%), despite the prominence of clarithromycin as a major antimicrobial agent in standard therapy [19]. Resistance to metronidazole and levofloxacin, which are used in alternative *H. pylori* eradication therapies, was also high (above 26%). Against 43 clarithromycin-resistant strains, the MIC$_{50}$ and MIC$_{90}$ of gentamicin and netilmicin were lower than those of other antibiotics, except amoxicillin, which is a component of the standard therapy. The overall resistance rate of antimicrobial agents to the 43 clarithromycin-resistant strains was higher than that of those of the total *H. pylori* strains. Among aminoglycosides, gentamicin and netilmicin had a lower MIC than tobramycin. The MIC$_{90}$ and MIC$_{90}$ of gentamicin and netilmicin were lower than that of metronidazole, tetracycline, and levofloxacin, which are alternative therapies for *H. pylori* eradication [19].

Generally, the MIC against most gram-negative bacteria for aminoglycosides is not below 1–4 mg/L [27, 28]. The resistance breakpoint against *Enterobacteriaceae* and *Pseudomonas* spp. was >4–16 mg/L. The breakpoints of aminoglycosides against *H. pylori* have not been previously studied; therefore, we conservatively assumed a strict resistance breakpoint of >1 mg/L for the estimation of the resistance rate. At a strict resistance breakpoint of >1 mg/L, gentamicin and netilmicin had lower resistance rates than the other antimicrobial agents currently used in *H. pylori* therapies; amoxicillin had the second-lowest resistance rate and this results were statistically significant (Fig. 1, Table 4).

Currently, smectite is expected to play a role as a drug delivery system, in addition to its original role as an antidiarrheal agent [22, 29–31]. Thus, gentamicin and netilmicin, which have low MICs for *H. pylori*, may be considered as good candidates for a smectite hybrid complex aimed at *H. pylori* eradication. Our results present a novel alternative therapy for *H. pylori* eradication by reporting the latest MIC of aminoglycosides against recently identified *H. pylori*. Aminoglycoside-intercalated smectite hybrids are expected to emerge as a novel standard therapy for the eradication of *H. pylori*, which is resistant to conventional antibiotics and has a low eradication rate.

And the serum concentration is a very important factor in the use of aminoglycosides. The S-GEN formulation proposed in our previous study, is a novel method of topical application to the stomach wall by oral administration. Because the aminoglycoside is not absorbed, its serum concentration is not significant. This is the key point where hybrid formulation differs from conventional *H. pylori* eradication therapy.

We have already reported the results of animal experiments using *H. pylori* standard strains for in vivo activity at low pH conditions [22]. After confirming the in vivo activity of *H. pylori* standard strains, this paper tried to establish evidence that it is applicable not only to standard strains but also to recent clinical isolated strains. In other words, it was necessary to check whether the MIC for the recent clinical strains remained still low as in the past, and whether it could be commercialized in clinical practice.

This study has some limitations with respect to the progression to in vivo studies and applications in real practice. First, the microbiological activity of aminoglycosides is pH-dependent. In an in vitro study, for example, the MIC of aminoglycosides was increased at pH <6.5 [32]. Thus, they should be applied with antacid drugs to overcome gastric acidity.
When we added PPI to S-GEN in our previous animal studies, we found therapeutic effects similar to those of the standard therapies [22]. Second, direct comparison of MIC values is controversial. It is necessary to consider the pharmacokinetics and pharmacodynamics of each antimicrobial agent in the interpretation of MICs; furthermore, clinical studies should be considered. Third, aminoglycosides can cause serious adverse effects such as nephrotoxicity and ototoxicity [20, 33]. Further research is needed to overcome these drawbacks and localised therapy in clinical studies with aminoglycosides-smectite hybrid complex needs to prove that they do not cause such adverse effects.

In conclusion, our data demonstrated that gentamicin and netilmicin still have low MIC values against recently isolated H. pylori, even though many intravenous and intramuscular aminoglycosides have been used for decades. These MIC values are believed to be sufficient to eradicate H. pylori by using smectite hybrid complex coating forms as localised therapy. Additional clinical studies should confirm that localised aminoglycoside treatment with a smectite drug delivery system is effective against H. pylori, as shown in the animal studies.

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SUPPLEMENTARY MATERIAL

Supplementary material can be found with this article on-line https://icjournal.org/src/sm/ic-5110-s001.xls.

Supplementary Table 1
Minimum inhibitory concentrations of antibiotics for Helicobacter pylori strains (n = 222)

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