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ORIGINAL ARTICLE

Comparative *in vitro* antimicrobial susceptibility and synergistic activity of antimicrobial combinations against *Helicobacter pylori* isolates in Taiwan

Aristine Cheng [a,b], Wang-Huei Sheng [a,*], Jyh-Ming Liou [a], Hsiu-Po Wang [a], Ming-Shiang Wu [a], Jaw-Town Lin [a,c], Shan-Chwen Chang [a]

[a] *Department of Internal Medicine, National Taiwan University Hospital and College of Medicine, Taipei, Taiwan*
[b] *Department of Internal Medicine, Far Eastern Memorial Hospital, Taipei, Taiwan*
[c] *Department of Internal Medicine, E-Da Hospital and I-Shou University, Kaohsiung, Taiwan*

Background: Antimicrobial resistance is a major determinant of *Helicobacter pylori* treatment failures. We conducted a population-based survey to monitor changing antimicrobial susceptibility of *H. pylori* isolates in Taiwan, with a focus on combinatorial effects of synergism and the influence of acidity.

Methods: *H. pylori* isolates from endoscopic specimens taken from patients enrolled at two medical centers were obtained between January 2008 and December 2009. Minimum inhibitory concentrations (MICs) were determined by agar dilution and *E*test methods. Agar media of varying pH (pH 7.3, 6.0, or 5.0) were used to assess whether acidity influences the bactericidal effects of the agents tested. Time-kill assays were used to assess for synergism between different drug combinations.

Results: A total of 176 non-duplicate *H. pylori* isolates from endoscopic specimens were tested. The following MIC_{90} (mg/L) (susceptible) results were obtained at neutral pH 7.3: amoxicillin, 0.25 (100%); tetracycline, 0.5 (100%); metronidazole, 32 (67.6%); clarithromycin, 0.25 (90.3%); ciprofloxacin, 1 (92.0%); gemifloxacin, 0.5 (94.9%); levofloxacin, 1 (93.2%); and moxifloxacin, 1 (91.5%). A decrease in pH from 6.0 to 5.0 significantly decreased the antimicrobial activity of levofloxacin and moxifloxacin against *H. pylori*. For clarithromycin-susceptible

isolates, levofloxacin combined with clarithromycin provided both synergistic and bactericidal effects. For clarithromycin-resistant isolates with amoxicillin hypersusceptibility (MIC <0.01 mg/L), levofloxacin with amoxicillin or minocycline had at best additive effect but no bactericidal effects.

Conclusion: Amoxicillin, tetracycline, clarithromycin, and fluoroquinolones, but not metronidazole, showed good *in vitro* anti-*H. pylori* activity (>90% susceptible). Synergism was only observed for clarithromycin-susceptible isolates. Acidity adversely influenced the antimicrobial activity of levofloxacin against *H. pylori*.

Introduction

Helicobacter pylori is one of the most common causes of chronic bacterial infections worldwide.[1] Its prevalence is significantly higher among patients with duodenal (>95%) and gastric ulcers (80%) than in asymptomatic healthy volunteers (50%),[1–3] and its role in the pathogenesis of peptic ulcer diseases, functional dyspepsia, and gastric cancer is well recognized.[4–7] Consequently, eradication of *H. pylori* remains a primary goal for alleviating peptic ulcer disease and preventing associated gastric malignancies.[8,9]

Over the past three decades, numerous regimens for *H. pylori* eradication have been tested.[10,11] Triple and quadruple regimens containing at least two antibiotics appear most successful and have been recommended by the Maastricht IV guidelines for standard therapy.[12] Among the most popular first-line regimens are clarithromycin-based triple therapy, containing a proton-pump inhibitor (PPI) combined with amoxicillin (1 g) and clarithromycin (500 mg) twice daily for 7 days, and a four-drug combination consisting of a PPI combined with bismuth (120 mg) and tetracycline (500 mg) four times daily and metronidazole (500 mg) three times daily. Understandably, *H. pylori* strains resistant to clarithromycin, amoxicillin, metronidazole, and levofloxacin have become increasingly prevalent worldwide, including Taiwan.[13,14] However, antimicrobial susceptibility patterns have not been uniformly predictable.[15] Resistance rates reported vary from 10% to 90% for metronidazole, from 0% to 45% for clarithromycin, from 0% to 33% for amoxicillin, from 5% to 59% for tetracycline, and from 6% to 21% for levofloxacin.[13,16–18]

Since antibiotic resistance is the major determinant of treatment failure,[19] there is an ongoing need to define resistance rates locally and to monitor these changing susceptibility patterns to guide optimal therapy. Hence, we undertook a population-based survey of the antimicrobial susceptibility of *H. pylori* isolates collected from northern and southern regions in Taiwan.

Materials and methods

Patients

Between January 2008 and December 2009, we enrolled patients undergoing upper gastrointestinal endoscopy for isolation of *H. pylori* from endoscopic specimens at the National Taiwan University Hospital (NTUH) and at E-Da Hospital in northern and southern Taiwan, respectively. Only non-duplicate *H. pylori* isolates were studied. *H. pylori* samples were recovered from antral biopsies from patients positive for a *Campylobacter*-like-organisms test (Pronto-Dry, Medical Instruments, Brignais, France). Demographic characteristics including age, sex, underlying systemic diseases, symptoms requiring endoscopic examination, and clinical diagnosis were recorded. To exclude secondary resistance of the strains, patients who had taken antimicrobial agents within 3 months prior to endoscopic examination or had a history of previous antimicrobial therapy to eradicate *H. pylori* infection were excluded. This study was approved by the Ethics Committee of NTUH (IRB-NTUH-REO No. 200702009). Informed consent was obtained from all patients.

Biopsy specimens and culture for *H. pylori*

Gastric biopsy specimens were stored in cysteine freezing medium at $-80\ °C$ before processing in a sterile tissue grinder with heat-inactivated fetal bovine serum. Samples were inoculated onto blood agar (tryptic soy agar with 5% sheep blood), onto chocolate agar, and onto *Brucella* agar containing 10% horse blood, trimethoprim, vancomycin, and polymyxin B. All cultures were incubated at $37\ °C$ under microaerophilic conditions and high humidity (12% CO_2, 98% humidity) for up to 10 days. Positive cultures were usually identified after 3–5 days of incubation. Isolates were identified as *H. pylori* on the basis of positive catalase, oxidase, and urease reactions; typical uniform, small, translucent colonies; curved Gram-negative bacilli on Gram-stained smears; susceptibility to cephalothin (30 μg); and resistance to nalidixic acid (30 μg).

Antimicrobial susceptibility tests for *H. pylori* on agar of different pH

Minimal inhibitory concentrations (MICs) were determined by agar dilution methods according to the Clinical and Laboratory Standards Institute (CLSI) as previously described.[20,21] In brief, *H. pylori* was inoculated onto antibiotic-containing Mueller–Hinton agar supplemented with 5% defibrinated sheep blood. The MIC of each antibiotic was determined after 72 h of incubation. *H. pylori* ATCC 43504 was used as the quality control strain.

In addition, the susceptibility of the isolates was tested using the Etest method (Epsilometer test; AB Biodisk, Solna, Sweden) as recommended by the British Society for

Antimicrobial Chemotherapy (BSAC) with two minor modifications.[22] In brief, colonies from a 2-days culture on a blood agar plate were suspended in sterile distilled water to an adjusted McFarland 3 standard. *Brucella* chocolate agar with 7% sheep blood was used instead of Mueller—Hinton agar with 5—10% horse blood. The 140-mm-diameter agar plates were inoculated by confluent swabbing of the surface with the adjusted inoculum suspensions. Five *E*test strips were aseptically placed onto the dried surface of each inoculated agar plate. The plates were incubated at 37 °C instead of 35 °C under microaerophilic conditions for 3 days. The MICs were read after 72 h of incubation on the basis of the intersection of the elliptical zone of complete growth inhibition with the MIC scale on the *E*test strip according to the manufacturer's instructions.[23] The following control strains were used: ATCC 43504, ATCC 43526, and ATCC 43579. Each strain was tested at least twice using *E*test strips from different batches. Testing was performed using agar culture media at different pH values (neutral pH 7.3 ± 0.1, and acidic pH 6.0 and 5.0).

The breakpoints used to classify strains as susceptible or resistant according to MIC are listed in Table 1. The breakpoints for clarithromycin were interpreted according to CLSI recommendations. The breakpoints for amoxicillin, metronidazole, and tetracycline were interpreted according to the BSAC recommendations (not published by CLSI). Quinolones are not standardized by the CLSI nor BSAC, so we used the recommendations of the Societé Française de Microbiologie, in accordance with other authors.[23]

Antimicrobial synergism of different combinations of antibiotics

Time-kill curves for different levofloxacin-based combinations were plotted to investigate for synergism against *H. pylori* isolates. Time-kill analyses were performed for two clarithromycin-susceptible and four clarithromycin-resistant *H. pylori* isolates. Each *H. pylori* isolate was subcultured twice on blood agar plates and a bacterial suspension of 10^8 colony-forming units (CFU)/mL was prepared based on a 1.0 McFarland standard. Aliquots of Mueller—Hinton broth with 5% lysed horse blood were inoculated with the bacterial suspension to yield a final suspension of 5×10^5 CFU/mL. The antimicrobial agents tested for synergism were added to yield concentrations

0.5 times their MIC. Control experiments without active compounds were conducted simultaneously. The final volume for each bacterium—drug concentration was 10 mL and samples were kept in a shaking water bath at 35 °C. An aliquot was taken from each sample at 0, 3, 6, and 24 h after addition of the antimicrobial combination. Multiple 1:10 dilutions were made in saline and were subcultured onto blood agar plates and incubated under 5% CO_2 for 18—24 h. Studies were repeated three times on consecutive days and time-kill data are presented as mean ± standard deviation. At each time interval, the log_{10} value of the viable colony count was determined. The lower limit of quantification in this model was 10 CFU/mL. Synergy of an antimicrobial combination was defined as a ≥ 2 log_{10} decrease in CFU/mL compared with its more active constituent. Bactericidal activity was defined as a minimum 3 log_{10} reduction in bacterial count at 24 h.[24]

Results

A total of 176 *H. pylori* isolates from endoscopic specimens were collected from 176 patients, 129 of whom were enrolled at the NTUH site and 47 patients at the E-Da site. MIC_{50} and MIC_{90} values for the eight antimicrobial agents tested are shown in Table 1. Amoxicillin and tetracycline showed good *in vitro* activity against the isolates, but the MIC range for metronidazole was very wide. The MIC distributions were lower for newer than for older quinolones, with gemifloxacin on the far left and ciprofloxacin on the far right (Figs. 1 and 2).

Antimicrobial susceptibility test results according to agar pH are shown in Table 2. When the acidity of the environment was increased from pH 7.0 to pH 6.0, the MIC_{90} for ciprofloxacin increased significantly across breakpoints. When the pH was further decreased from 6.0 to 5.0, the MIC_{90} for levofloxacin increased significantly across breakpoints, whereas smaller elevations in the MIC_{90} for amoxicillin, clarithromycin, tetracycline, moxifloxacin and gemifloxacin and in the MIC_{50} for metronidazole were observed.

Six *H. pylori* isolates with varying clarithromycin MICs, including two clarithromycin-susceptible isolates (HP-311, HP-246) and four clarithromycin-resistant isolates (HP-109, HP-103, HP-143, HP-82), were randomly selected for *in vitro* antimicrobial synergy testing (Table 3). Drug combinations of levofloxacin and clarithromycin,

Table 1 Minimum inhibitory concentration for various antimicrobial agents tested against 176 *H. pylori* isolates at neutral pH 7.3

Antimicrobial agent	Inhibitory concentration (mg/L)			Resistance (%)	Breakpoint criteria (mg/L)	
	MIC_{50}	MIC_{90}	MIC range		Susceptible	Resistant
Amoxicillin	0.03	0.25	0.008—0.5	0.0	≤1	≥2
Tetracycline	0.125	0.5	0.008—1	0.0	≤2	≥4
Metronidazole	4	32	0.03—128	32.4	≤4	≥8
Clarithromycin	0.06	0.25	0.008—>16	9.7	≤0.25	≥1
Ciprofloxacin	0.5	1	0.016—>16	8.0	≤1	≥2
Gemifloxacin	0.125	0.5	0.008—8	5.1	≤1	≥2
Levofloxacin	0.5	1	0.03—>16	6.8	≤1	≥2
Moxifloxacin	0.5	1	0.008—>16	8.5	≤1	≥2
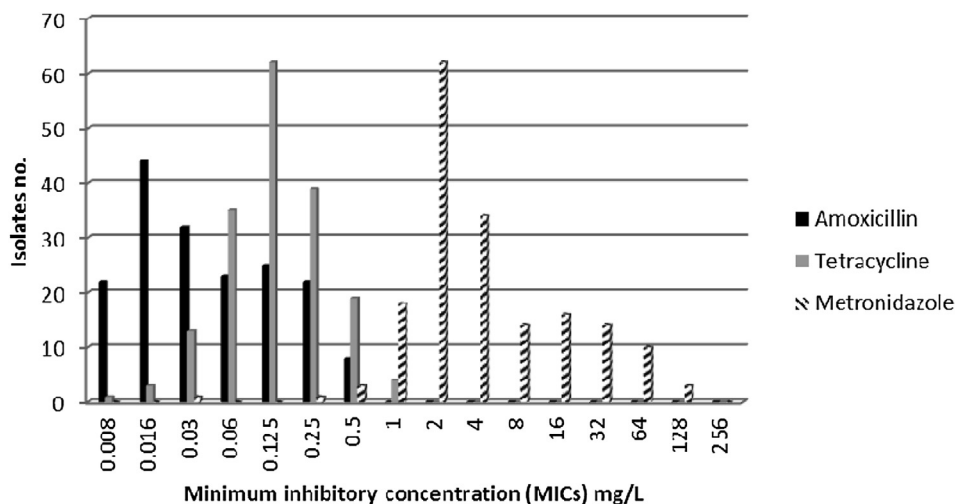
Figure 1. Minimum inhibitory concentration distributions for the 176 *H. pylori* isolates tested against amoxicillin, metronidazole, and tetracycline.
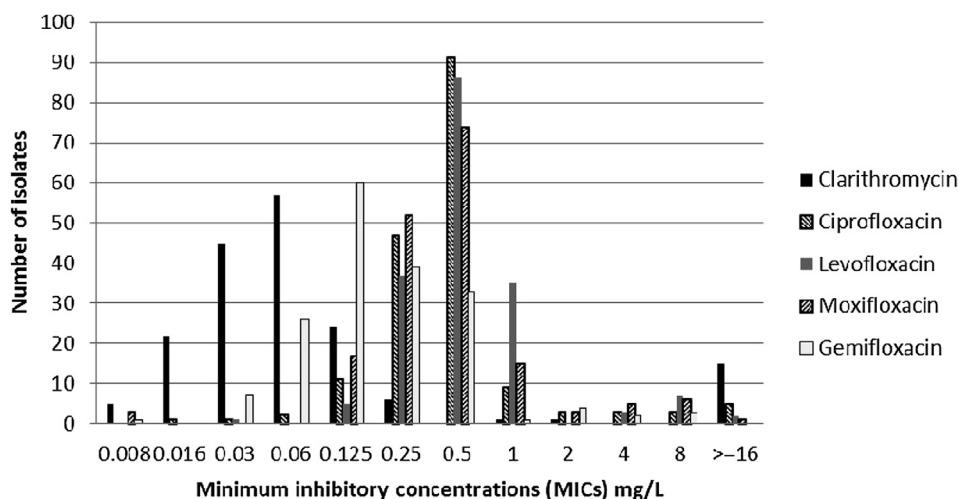
levofloxacin and amoxicillin, and levofloxacin and minocycline were tested. Time-kill assays for isolates susceptible to clarithromycin revealed bactericidal and synergistic effects for clarithromycin combined with levofloxacin (Fig. 3). However, for *H. pylori* isolates with clarithromycin resistance, additive but not synergistic or bactericidal effects were observed for levofloxacin + amoxicillin or levofloxacin + minocycline combinations. The benefit of adding a second drug to amoxicillin for clarithromycin-resistant isolates was more pronounced when the amoxicillin MIC for the isolates was <0.01 mg/L (Fig. 4).

Discussion

Our investigation revealed that amoxicillin, tetracycline, clarithromycin, and fluoroquinolones still show good *in vitro* anti-*H. pylori* activity in Taiwan. An increase in acidity significantly reduced antimicrobial susceptibility to fluoroquinolones. Synergism or an additive effect of

levofloxacin combinations with clarithromycin, amoxicillin, or minocycline was highly dependent on the susceptibility of *H. pylori* isolates to clarithromycin. The present study provides important information regarding the prevalence of drug-resistant *H. pylori* in Taiwan.

We found that approximately one-third of pre-treatment *H. pylori* isolates were resistant to metronidazole and one-tenth to clarithromycin. While our updated resistance rates to metronidazole show a rebound from the interim trough reported for this region (from 25.4% to 32.4%),[25] the rates for clarithromycin have remained stable over the last decade.[13] Thus, more frequent *H. pylori* eradication failures may be anticipated when metronidazole is used in first-line regimens in areas where metronidazole resistance is prevalent, such as Taiwan and Tunisia.[11,20,26] For the increasing yet fluctuating patterns of antimicrobial resistance of *H. pylori* isolates, susceptibility testing (either phenotypic or genotypic) of *H pylori* isolates prior to empirical treatment would be optimal in terms of

Figure 2. Minimum inhibitory concentration distributions for the 176 *H. pylori* isolates tested against clarithromycin, ciprofloxacin, levofloxacin, moxifloxacin, and gemifloxacin.

Table 2 Antimicrobial susceptibility tests performed in acidic agar culture media (pH 5.0 and 6.0)

Antimicrobial agent	Inhibitory concentration (mg/L)					
	pH 6.0			pH 5.0		
	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range
Amoxicillin	0.125	0.125	0.016−0.25	0.19	0.25	0.125−0.5
Tetracycline	0.09	0.125	0.03−0.25	0.19	0.5	0.06−0.5
Metronidazole	2	16	2−32	4	16	2−32
Clarithromycin	0.5	1	0.25−>16	0.5	1.5	0.25−>16
Ciprofloxacin	0.75	2	0.25−16	0.75	2	0.5−>16
Gemifloxacin	0.25	0.5	0.125−4	0.5	0.75	0.125−8
Levofloxacin	0.5	1	0.5−8	1.5	2	0.5−8
Moxifloxacin	1	2	0.5−8	4	6	0.5−16

achieving higher eradication rates without fueling resistance.[12,14,20]

However, since pretreatment antimicrobial susceptibility testing is not widely available in Taiwan to guide initial therapy, physicians should note that metronidazole resistance has been consistently associated with female sex.[17,27] In addition, since metronidazole resistance rates vary according to overall metronidazole consumption rates, its future role in empiric *H. pylori* therapy will continue to evolve depending on changing susceptibility rates.

Despite its lack of novelty, amoxicillin showed good *in vitro* activity, indicating that amoxicillin should still be the first choice for *H. pylori* eradication in Taiwan. Clarithromycin resistance rates of <15−20% indicate that the empiric clarithromycin-based triple therapy proposed by the Maastricht IV/Florence consensus remains applicable to our population.[12]

In this study we also observed minimal *in vitro* resistance to older shelved drugs such as tetracycline and to ciprofloxacin. These drugs may potentially be used as salvage therapy for patients for whom the above clarithromycin-based standard first-line therapy fails.[28] In agreement with a previous report,[29] we found that newer generation fluoroquinolones such as gemifloxacin were superior to other fluoroquinolones against clinical *H. pylori* isolates. Whether gemifloxacin provides better clinical outcomes than other fluoroquinolones through fewer iterative failures warrants further studies.

The effect of pH changes on the activity of antimicrobial agents, in particular for the newer quinolones, has not been

Table 3 Antimicrobial susceptibility of six *H. pylori* strains randomly selected to represent the clarithromycin susceptibility range

H. pylori strain	Antimicrobial susceptibility (mg/L)		
	Amoxicillin	Clarithromycin	Levofloxacin
HP-311	0.006	0.006	0.5
HP-246	0.5	0.125	0.5
HS-109	0.06	>16	0.5
HS-103	0.016	>16	8
HS-143	0.008	>16	1
HS-82	0.008	>16	0.25

well described for *H. pylori*.[30−32] Our data demonstrate that an increase in pH enhanced the bactericidal activity of all antibacterial agents against *H. pylori*, albeit to varying extents. However, the influence of pH on MIC breakpoints appeared most marked for selected fluoroquinolones, namely for ciprofloxacin and levofloxacin, wherein a categorical change from susceptible to resistant is observed when the acidity levels increased from a pH 7.0 to 6.0 and from pH 6.0 to pH 5.0, respectively. Changes in gastric acidity can also affect the expression of bacterial target sites and survival.[33,34] Together, these effects observed *in vitro* contribute to the potentiating *in vivo* effects of concomitant acid suppression therapy for *H. pylori* eradication. A lower pH did not appreciably alter the bactericidal effects of metronidazole, as previously described.[30,31,34]
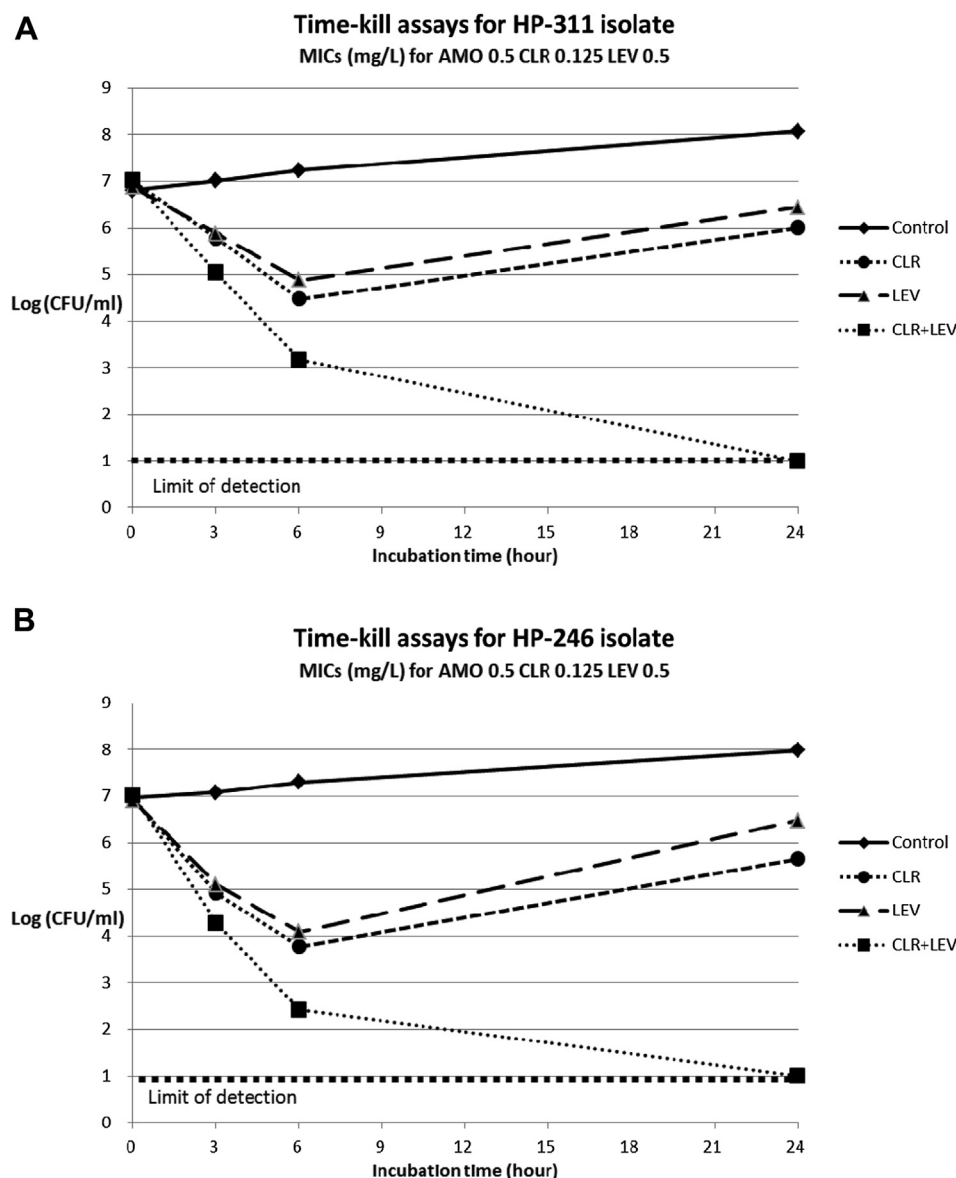
In Taiwan, current eradication regimens available for *H. pylori* include triple drug combinations comprising a proton pump inhibitor and two antibiotics, amoxicillin plus metronidazole, clarithromycin plus metronidazole, or clarithromycin plus amoxicillin, with eradication rates of 70%, 79%, and 89%, respectively.[35] Although it may be possible to explain these eradication rates by considering primary resistance rates to individual agents alone, we used time-kill assays to explore the potential for synergism between various agents in levofloxacin-based combinations.

Previous studies have not reliably demonstrated *in vitro* synergy between various combinations of amoxicillin, clarithromycin, and metronidazole with a proton-pump inhibitor for antibiotic-resistant strains of *H. pylori*.[36,37] To the best of our knowledge, there are no reports in the English literature that clarify the interactions of levofloxacin with a second antibiotic, especially for clarithromycin-resistant strains, for which levofloxacin might play a role in salvage therapy. Our study demonstrated that clarithromycin susceptibility predicted good antimicrobial synergism for the combination of levofloxacin (instead of amoxicillin) and clarithromycin. However, for *H. pylori* isolates with clarithromycin MIC values >1 mg/L, levofloxacin combined with either amoxicillin or minocycline was neither synergistic nor bactericidal. This result supports findings by Gotoh et al., who observed that when a strain was resistant to one drug (especially clarithromycin) in a combination, no synergism was detected.[37]

A

Time-kill assays for HP-311 isolate
MICs (mg/L) for AMO 0.5 CLR 0.125 LEV 0.5

B

Time-kill assays for HP-246 isolate
MICs (mg/L) for AMO 0.5 CLR 0.125 LEV 0.5

Figure 3. Time-kill assays for clarithromycin (CLR)-susceptible *H. pylori* strains (A) HP-311 and (B) HP-246. There is good antimicrobial synergism and bactericidal effects for levofloxacin (LEV) and CLR combinations against these two strains.

Moreover, addition of amoxicillin to levofloxacin has no effect for isolates with amoxicillin MIC values >0.01 mg/L.

Previous time-kill studies predicted synergism if an isolate is sensitive to both antibiotics used in the combination.[37] However the most interesting finding from our time-kill assays was that given clarithromycin resistance, despite dual sensitivity to amoxicillin and levofloxacin, this combination was not synergistic. Amoxicillin rather than levofloxacin MIC values predicted whether levofloxacin combinations have an additive effect or not.

A levofloxacin-containing cocktail or sequential therapy may replace standard triple therapy in areas with high (>15—20%) clarithromycin resistance.[12] However, for clarithromycin-resistant isolates, we observed indifferent or at best additive effects using levofloxacin-containing combinations. Hence, concurrent levofloxacin-based therapy would not be expected to be superior to sequential rescue therapy. Our group confirmed this in a clinical trial of

sequential salvage therapy consisting of esomeprazole 40 mg and amoxicillin 1 g for the first 5 days, followed by esomeprazole 40 mg, levofloxacin 250 mg, and metronidazole 500 mg for another 5 days (all given twice daily) in patients for whom standard triple therapy failed. Eradication rates were >95% using this sequential levofloxacin-containing regimen in the absence of metronidazole resistance [26].

Our results suggest that both amoxicillin and clarithromycin remain pivotal in the therapy of *H. pylori*-associated disease. The susceptibilities to these agents define the synergism of levofloxacin-based antimicrobial and PPI combinations. Hence, pre-salvage treatment susceptibility testing for clarithromycin, amoxicillin, and metronidazole to define resistance and/or multiple drug resistance patterns of *H. pylori* isolates should be considered as a more advanced treatment approach at the individual and population level.
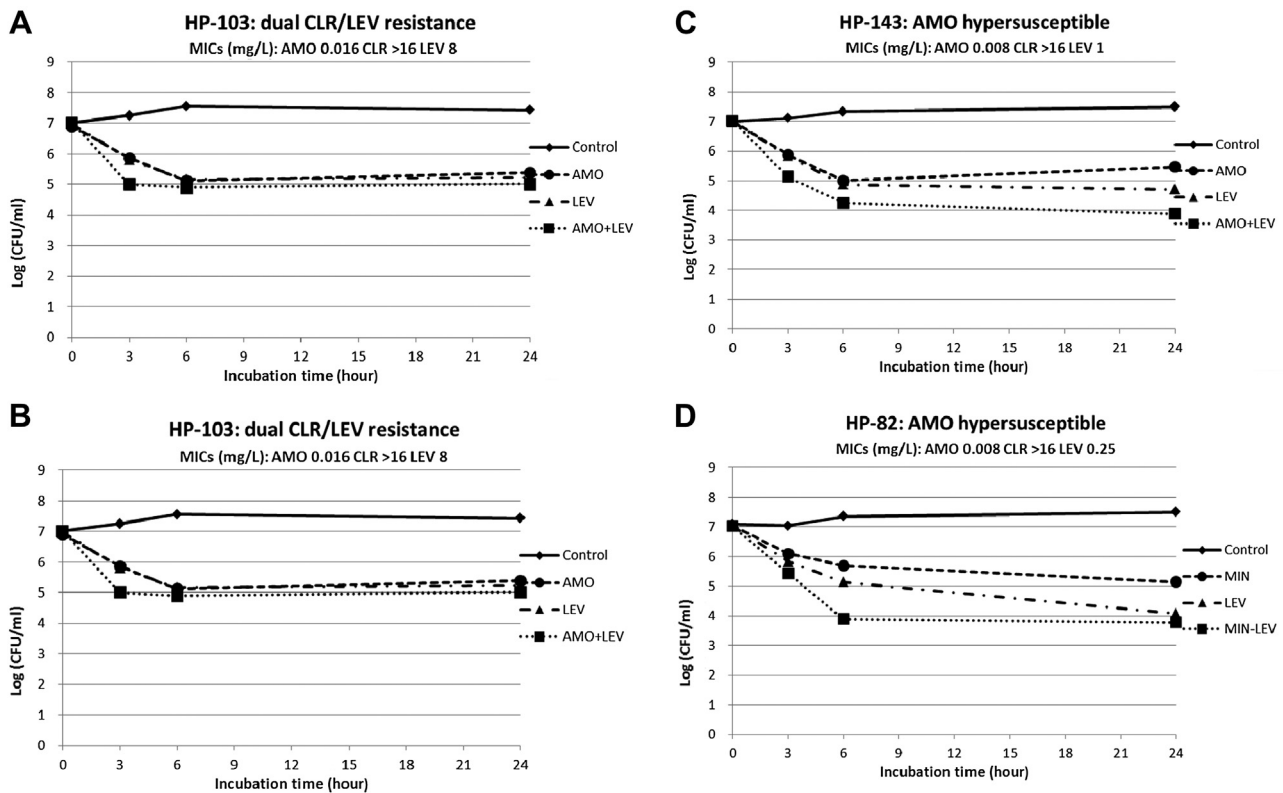
Figure 4. Time-kill assays for clarithromycin (CLR)-resistant *H. pylori* strains HP-109, HP-103, HP-143, and HP-82. None of the levofloxacin (LEV)-based combinations demonstrated a bactericidal effect for CLR-resistant isolates. No synergism was observed for isolates, with amoxicillin (AMO) MICs >0.01 mg/L regardless of LEV susceptibility (3A, 3B). Pronounced additive effects existed between LEV and AMO or minocycline (MIN) combinations for isolates, with AMO MICs <0.01 mg/L (3C, 3D).

Our study supports the continued use of amoxicillin in combination with clarithromycin as first-line therapy for *H. pylori* in urban Taiwan. Quinolones should be reserved as second-line therapy. Since all antimicrobial activities were enhanced at higher pH values, the two other prerequisites for effective treatment are adequate acid suppression and good drug compliance. Although additive effects were observed when levofloxacin was combined with a second effective antibiotic, no *in vitro* synergism existed to support concurrent sequential regimens to eradicate clarithromycin-resistant *H. pylori*.

Conflicts of interest statement

All authors declare that they have no conflicts of interest.

Acknowledgments

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