

Original Article

***Helicobacter pylori* antibiotic susceptibility patterns in Bangladesh: Emerging levofloxacin resistance**Hafeza Aftab^{1,2}, Muhammad Miftahussurur^{1,3}, Phawinee Subsomwong¹, Faruque Ahmed², AK Azad Khan⁴, Yoshio Yamaoka^{1,5}¹ Department of Environmental and Preventive Medicine, Oita University Faculty of Medicine, Yufu, Japan² Department of Gastroenterology, Dhaka Medical College and Hospital, Dhaka, Bangladesh³ Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia⁴ Department of Gastroenterology, BIRDEM, Dhaka, Bangladesh⁵ Department of Medicine, Gastroenterology and Hepatology Section, Baylor College of Medicine, Houston, Texas, United States**Abstract**

Introduction: The most recent study to report *Helicobacter pylori* antibiotic resistance rates in Bangladesh was published 15 years ago and did not include levofloxacin. We therefore aimed to determine the current antibiotic susceptibility of *H. pylori* to amoxicillin, clarithromycin, metronidazole, tetracycline and levofloxacin in Bangladesh.

Methodology: This study included 133 consecutive patients who underwent endoscopy examination at Dhaka Medical College in November 2014. The serial two-fold agar dilution method was used to determine the minimum inhibitory concentrations of the five antibiotics.

Results: Among 56 cultured strains, *H. pylori* showed high rates of resistance to clarithromycin and metronidazole (39.3% and 94.6%, respectively). Moreover, levofloxacin showed an emerging antimicrobial resistance pattern (66.1%), which was higher in patients with gastritis than that in those with peptic ulcers ($p = 0.02$). The resistance rate of levofloxacin was significantly higher in patients living in Dhaka city compared to those living in the village ($p = 0.049$). However, amoxicillin and tetracycline resistance rates were very low. Resistance to both metronidazole and levofloxacin was most commonly observed.

Conclusions: The rates of resistance to clarithromycin, metronidazole, and levofloxacin were high in Bangladesh, which suggests that triple therapy based on these drugs may not be useful as first-line therapies in Bangladesh. Alternative strategies such as furazolidone-based triple therapy, bismuth-based quadruple therapies, or sequential therapy may be more effective for patients in Bangladesh.

Key words: Bangladesh, drug resistance, *Helicobacter pylori*.*J Infect Dev Ctries* 2016; 10(3):245-253. doi:10.3855/jidc.7713

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Copyright © 2016 Aftab *et al.* This is an open-access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.**Introduction**

Successful treatment of *Helicobacter pylori*, the Gram-negative bacterium responsible for infections affecting more than half of the world's population, results in pathogen eradication and often also cures and prevents development of associated diseases, including chronic gastritis, peptic ulcer diseases, gastric cancer, and mucosa-associated lymphoid tissue lymphoma [1]. First-line, alternative first-line, second-line, and even third-line therapies for *H. pylori* eradication have been proposed. According to current guidelines, a triple therapy containing a proton pump inhibitor and two antibiotics, amoxicillin (AMX) and clarithromycin (CAM) or metronidazole (MNZ), remains the standard first-line regimen for treatment of *H. pylori* infection [2-5]. However, in recent years, the efficacy of these legacy triple regimens has been seriously challenged

and eradication rates below 70% have been reported in many countries [6]. Moreover, some regions in Asia show patterns of emerging antimicrobial resistance [7]. Local antibiotic resistance screening and selection of appropriate first-line regimens are essential to combat *H. pylori* antibiotic resistance [7]. These steps could prevent repeated treatment courses that may result in multiple side effects and spread of secondary antibiotic resistance [6].

Bangladesh is a country in South Asia with over 160 million people, making it the world's eighth-most populous country. The prevalence of *H. pylori* infection is reportedly high (60.2%), and the prevalence rate is similar to that noted in developing countries (53.8% among those aged 12–19 years), which is thought to be related to overcrowding and poor sanitary conditions [8]. The *H. pylori* re-infection rate in Bangladeshi is

markedly higher than in Western countries, and duodenal ulcer relapse is clearly related to *H. pylori* reinfection [9]. The neighboring countries, India and Pakistan, have high CAM, AMX and MNZ resistance rates [7]. Previous studies have reported CAM, MNZ, AMX and tetracycline (TCN) resistance rates of 58.8%, 83.3%, 72.5%, 53.8%, and 36.0%, 89.0%, 37%, 12.0%, in India and Pakistan, respectively [10,11]. Levofloxacin (LVX)-based triple regimens or bismuth-based quadruple therapy has therefore been suggested to be most effective in this region. Although a previous study reported antibiotic resistance rates in Bangladesh, it was performed approximately 15 years ago (1999-2001) [12]. Moreover, the previous study did not measure the resistance rate for LVX, which has recently been used in second-line regimens and as a rescue treatment for *H. pylori* eradication. The previous study reported rates of resistance to CAM, AMX, MNZ and TCN to be 10%, 6.6%, 77.5%, and 15%, respectively [12]. The standard CAM-based triple regimen for 7-14 days is still the first option for eradicating *H. pylori* in Bangladesh, and LVX-based is the second line treatment [13]. Because antibiotic resistance is increasing worldwide [14,15], it is critical to examine current drug resistance rates in Bangladesh. In this study, we aimed to determine the antibiotic susceptibility of *H. pylori* to CAM, MNZ, AMX, TCN and LVX in Bangladesh.

Methodology

Patients and H. pylori

This study included 133 consecutive patients (61 male and 72 female; age range, 18 to 65 years; mean age, 35.2 ± 11.8 years) who underwent endoscopy examination at Dhaka Medical College in November 2014. Peptic ulcer disease, including gastric and duodenal ulcers was diagnosed by endoscopic observation, while chronic gastritis was determined by histologic examination. Exclusion criteria included a history of partial gastric resection, eradication therapy for *H. pylori* and treatment with bismuth-containing compounds, H₂-receptor blockers, or proton pump inhibitors (PPI) within four weeks before the study. Written informed consent was obtained from all participants and the protocol was approved by the Ethics Committee of Bangladesh Medical Research Council (BMRC), Dhaka, Bangladesh and Oita University Faculty of Medicine, Japan.

For *H. pylori* culture, antral biopsy specimens were homogenized and inoculated onto a commercial *Helicobacter* selective agar plate (Nissui Pharmaceutical Co. Ltd. Tokyo, Japan). The plates

were incubated for up to 10 days at 37°C under microaerophilic conditions (10% O₂, 5% CO₂, and 85% N₂). *H. pylori* isolates were identified based on colony morphology; Gram staining results and positive biochemical tests (reactions on oxidase, catalase and urease). Isolated strains were stored at –80°C in Brucella Broth (Difco, Franklin Lakes, USA) containing 10% dimethyl sulfoxide and 10% horse serum.

Antibiotic susceptibility testing

The serial two-fold agar dilution method was used to determine the minimum inhibitory concentrations (MICs) of CAM (Abbott Laboratories, Abbott Park, IL, USA), AMX, MNZ, TCN and LVX (all: Sigma Chemical Co., St Louis, MO, USA). Briefly, bacteria were subcultured on Mueller-Hinton II Agar medium (Becton Dickinson) supplemented with 10% defibrinated horse blood. The bacterial suspension, adjusted to be equivalent to a McFarland opacity standard of 3.0, was inoculated onto the plates. After 72 hours of incubation, the MIC of each antibiotic was determined. Quality control was performed using *H. pylori* ATCC 43504. The resistance breakpoints were determined as described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; available in <http://www.eucast.org/>). Strains were considered to be resistant for MICs >0.125 mg/L for AMX, 0.25 mg/L for CAM, 8 mg/L for MNZ, and 1 mg/L for TCN and LVX.

Statistical analysis

Discrete variables were tested using the chi-square test, while continuous variables were tested using the Mann-Whitney *U* and *t*-tests. A multivariate logistic regression model was used to calculate the odds ratios (OR) of the places of residence and antibiotic resistance. All determinants with *p* values < 0.10 were entered together into the full logistic regression model, and the model was reduced by excluding variables with *p* values > 0.10. The OR and 95% confidence interval (CI) were used to estimate risks. *P* values < 0.05 were considered statistically significant. The SPSS statistical software package version 18.0 (SPSS, Inc., Chicago, IL) was used for all statistical analyses.

Results

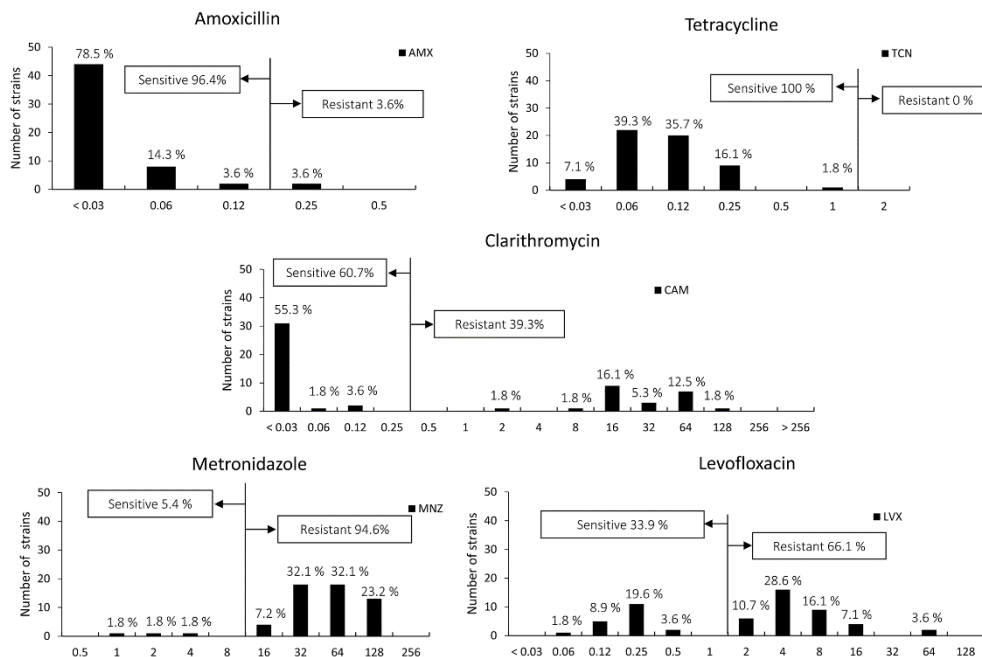
A total of 56 *H. pylori* strains were isolated: 38 from patients living in Dhaka city and 18 from the village outside Dhaka city. They were isolated from 26 male (age range, 18 to 56 years; mean age, 34.2 ± 11.6 years) and 30 female patients (age range, 19 to 65 years; mean

age 36.1±12.1 years). Among these patients, 53 had chronic gastritis and three had peptic ulcer diseases.

Interestingly *H. pylori* showed a higher rate of resistance to CAM and MNZ (22/56, 39.3% and 53/56, 94.6%; Table 1) than previously reported in Bangladesh [12]. Moreover, the LVX resistance rate also indicated emerging antimicrobial resistance (37/56, 66.1%), a similar phenomenon to that reported in India [11]. However, in contrast with other South Asian countries,

the AMX resistance rates were very low (2/56, 3.6%) and no TCN-resistant strains were observed. Overall, only one strain was susceptible to all tested antibiotics and 16 strains (28.6%) were resistant to one antibiotic. Although the number of strains isolated from patients with peptic ulcers was not sufficient for comparison, the rate of resistance to LVX was higher in patients with gastritis than in those with peptic ulcers (P = 0.02). The distribution of patient age and antimicrobial resistance

Figure 1. Distribution of antibiotic MIC values.



The resistance rates to clarithromycin, metronidazole, levofloxacin were high; in contrast with other South Asian countries, resistance rates to amoxicillin and tetracycline were very low.

Table 1. Antibiotic susceptibility of 56 *H. pylori* strains isolated in Bangladesh

Antibiotics	Number (%) of Resistant Patient Isolates				
	All patients (n = 56)	PUD (n = 3)	Gastritis (n = 53)	Male (n = 26)	Female (n = 30)
AMX	2 (3.6)	0 (0)	2 (3.8)	0 (0)	2 (6.7)
CAM	22 (39.3)	1 (33.3)	21 (39.6)	10 (38.5)	12 (40)
MNZ	53 (94.6)	3 (100)	50 (94.3)	25 (96.2)	28 (93.3)
TCN	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
LVX	37 (66.1)	0 (0)	37 (69.8)	16 (61.5)	21 (70)

Abbreviations: AMX, amoxicillin; CAM, clarithromycin; MNZ, metronidazole; TCN, tetracycline; LVX, levofloxacin; PUD, peptic ulcer disease.

Table 2. Distribution of antibiotic resistance in Bangladesh by age

Antibiotics	Number (%) per age group (years)					Total
	≤20	21-30	31-40	41-50	≥ 51	
Total	6	20	14	10	6	56
AMX	0 (0)	0 (0)	2 (14.3)	0 (0)	0 (0)	2
CAM	3 (50)	7 (35)	5 (35.7)	5 (50)	2 (33.3)	22
MNZ	5 (83.3)	18 (90)	14 (100)	10 (100)	6 (100)	53
TCN	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
LVX	2 (33.3)	16 (80)	9 (64.3)	5 (50)	5 (83.3)	37

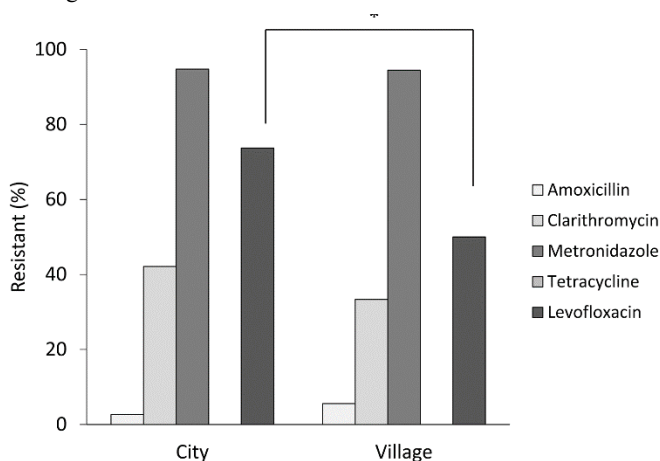
Abbreviations: AMX, amoxicillin; CAM, clarithromycin; MNZ, metronidazole; TCN, tetracycline; LVX, levofloxacin; PUD, peptic ulcer disease.

of the isolates is shown in Table 2. Antibiotic resistance did not differ among different age groups (P = 0.65, 0.99, 0.07, and 0.82 for AMX, CAM, MNZ and LVX, respectively).

The distribution of MIC values for each antibiotic is shown in Figure 1. *H. pylori* showed a high level of resistance (128 mg/L or more) to MNZ in 23.2% (13/56) of isolates, and CAM in 1.8% (1/56). We also compared resistance rates between the two geographical areas (Dhaka city and village). The resistance rate for LVX was significantly higher in people living in Dhaka city than among those living in the village (73.7% vs. 50.0%, P = 0.049; Table 3). Even after adjusting for age and sex using multivariate analysis, the resistance rate for LVX remained significantly higher in people living in Dhaka city (OR = 3.7; 95% CI: 1.08–13.2; P = 0.04) (Figure 2). In contrast, the resistance rates of AMX, CAM, and MNZ did not differ between these locations.

The distribution of multidrug-resistant strains is shown in Table 4. Among all strains, 28.6% (16/56) showed double-drug resistance to MNZ and LVX. Resistance to three antibiotics (CAM, MNZ, and LVX) was observed in 17 (30.4%) strains; however, only two strains were resistant to a combination of AMX, MNZ, and LVX. No differences were observed in clinical outcomes between single-drug and multidrug resistant infections (P = 0.06).

Figure 2. Distribution of antibiotic resistance in Dhaka city and village.



The resistance rate for levofloxacin (LVX) was significantly higher in people living in Dhaka city than in those living in the village (*).

Discussion

Our study of patients in Bangladeshi observed high *H. pylori* resistance rates to CAM and MNZ (39.3% and 94.6%), which are currently recommended as first-line therapeutics in many Asian countries [2-5]. CAM resistance has been shown to be associated with any one of three well-known point mutations in the 23S ribosomal RNA (rRNA) gene of *H. pylori*; these three mutations are responsible for more than 90% of CAM resistance cases in developed countries [16].

Table 3. Distribution of antibiotic resistance between city and village

N	City		Village	
	38		18	
Age	34.7 ± 12.5		36.2 ± 10.5	
Sex	Female	19 (50)	11 (61.1)	
	Male	19 (50)	7 (38.9)	
Disease	PUD	2 (5.3)	1 (5.6)	
	Gastritis	36 (94.7)	17 (94.4)	
Antibiotic	AMX	1 (2.6)	1 (5.6)	
	CAM	16 (42.1)	6 (33.3)	
	MNZ	36 (94.7)	17 (94.4)	
	TCN	0 (0)	0 (0)	
	LVX	28 (73.7)	9 (50)	

Abbreviations: AMX, amoxicillin; CAM, clarithromycin; MNZ, metronidazole; TCN, tetracycline; LVX, levofloxacin;

Table 4. Multidrug resistance patterns of *H. pylori* in Bangladesh

Resistance patterns	N (%)	
Double drugs		
CAM + MNZ	5	(8.9)
MNZ + LVX	16	(28.6)
Triple drugs		
AMX + MNZ + LVX	2	(3.6)
CAM + MNZ + LVX	17	(30.4)

Abbreviations: CAM, clarithromycin; MNZ, metronidazole; LVX, levofloxacin; AMX, amoxicillin.

Interestingly, the point mutations associated with CAM resistance in Asian countries differ from those in Europe and North America [17]. The T2183C and A2223G mutations have been frequently found to be the cause of observed CAM resistance, while the A2143G mutation, which has a much stronger impact than the A2142G and A2142C [18]. mutations responsible for 90% of primary CAM-resistant *H. pylori* cases isolated in Western countries [19], accounted only for 23% of resistant strains in Asia [19]. In Bangladesh, the T-to-C transition at position 2182 (T2182C) is the predominant mutation [20]. Antimicrobial resistance is considered a leading factor responsible for eradication therapy failure, in addition to lack of patient compliance, inadequate length of therapy, and high bacterial burden. This issue is of particular relevance with regard to CAM, which can induce a nearly 70% loss of effectiveness depending on macrolide susceptibility *in vitro* [21]. In addition, meta-analysis showed that use of triple therapy consisting of PPI, AMX, and CAM in cases of CAM resistance decreased the treatment efficacy by 66% [22]. CAM was first introduced in Bangladesh in the late 1990s, and has been widely used for eradication of *H. pylori*. The previous study reported that *H. pylori* resistance to antibiotics is linked to consumption of antibiotics in the region [23]. It is concordance with our results showing increased prevalence of CAM resistance compared to the approximately 10% rate reported in the previous study [12]. The Maastricht III guidelines on *H. pylori* infection management recommend substituting MNZ for CAM when resistance to this antibiotic exceeds 15% to 20% [24]. This second-line therapy was reported to be highly successful, with an eradication rate of more than 90% in Japan [25,26].

However, the resistance rate for MNZ is also very high in Bangladesh. The high incidence of MNZ-resistant strains observed in this study might be attributed to widespread over-the-counter use of this drug. MNZ is an inexpensive drug frequently used to treat not only *H. pylori* infection but also other infections such as intestinal parasites and periodontal and gynecologic diseases, which are common in developing countries such as Bangladesh [21, 23]. Although *in vitro* resistance to MNZ may not accurately reflect *in vivo* resistance and should be confirmed by measuring blood drug concentrations [27], regimens that include MNZ are not a preferable choice in populations with >40% resistance to this drug [28]. In Asia, only Japan, Thailand, and Malaysia have populations with <40% MNZ resistance [7], and preferentially use the PPI + CAM + MNZ regimen

recommended by the Maastricht III Consensus Report. However, regimens including MNZ are not suitable and should not be chosen as first-line treatment therapy in most other Asian countries, including Bangladesh.

Our findings showed a high prevalence of primary resistance to LVX (66.1%). LVX has recently been prescribed as a rescue drug to eradicate infection in patients with failed first-line therapy [29,30]. However, the incidence of LVX resistance seems to be increasing worldwide, which may reduce the efficacy of treatment with LVX-based regimens [31,32]. Therefore, according to European, Asia-Pacific, and American guidelines, LVX should be used in salvage therapy based on antibiotic susceptibility testing [2,24,33]. Interestingly, the LVX resistance rate was significantly higher in Dhaka city (73.7%) than in the village (50.0%). However, both areas showed high LVX resistance rates and LVX is not sufficiently effective to be included in treatment regimens in Bangladesh. In Europe, there was a significant association between outpatient quinolone use and the proportion of levofloxacin resistance [23]. Concordant with this data, since 1990, the ciprofloxacin (CIP) and ofloxacin (OFX) have been the antimicrobial drugs of choice for the treatment of multi drug resistance to typhoid fever, the highly endemic disease endemic in Bangladesh [34]. The high resistance rate to LVX in Bangladesh should be a cause of concern that resistance to other fluoroquinolones such as nalidixic acid, ciprofloxacin, and ofloxacin, which are commonly used in Bangladesh, may lead to cross resistance with LVX.

However, we observed no resistance to TCN, consistent with reports from other countries [35-38], but in contrast to studies from India and Pakistan [39-41]. This difference may in part be attributed to different usage of this antibiotic in various countries. Therefore, TCN-based or quadruple therapy including TCN may be a useful alternative first-line regimen in Bangladesh, as recommended in guidelines [2,24]. The TCN resistance mechanism has been characterized as a change in three contiguous nucleotides in the 16S rRNA gene (AGA 926-928RTTC) [42]. Interestingly, in contrast to other countries, resistance to TCN in Bangladesh is not related to mutations at positions 926 to 928 of the 16S rRNA gene [43]. In the present study, isolates from Bangladeshi patients had low resistance rate to AMX, similar to several countries in Asia, including China, Turkey, Bahrain, Malaysia, Bhutan, and Vietnam [7], but contrary to resistance rates reported in India and Pakistan [10, 11]. However, AMX is one of the most commonly used antibiotics in Bangladesh in recent years [12], and observation of

only two resistant strains is of some concern. Indeed, AMX resistance develops by genomic mutation in the *pbp1A* gene [16,44], and the resistance phenotype may be lost after storage or freezing [45,46]. However, Hu *et al.* concluded that resistance to AMX was stable before and after storage in -80°C for three months or years [47]. Further studies using AMX- and TCN-based regimens are necessary to determine the efficacy of these two antibiotics for *H. pylori* eradication in Bangladesh.

Multidrug resistance has recently appeared as a serious challenge in the fight against infections worldwide. *H. pylori* strains harboring triple or quadruple resistance can hinder the choice and success of eradication regimens. Our findings showed that 37.5% (21 strains) of the isolates were resistant to at least two antibiotics. Resistance to MNZ and LVX was most commonly observed (28.6%), and could be the reason for treatment failure in Bangladesh. In addition, we observed that 19 strains (33.9%) were resistant to three of the five antibiotics used in this study, especially CAM, MNZ, and LVX.

Bangladesh has a high prevalence of *H. pylori* infections; increased resistance to the antibiotics used to treat it might result in increased recurrence rates. With high morbidity and mortality rates due to *H. pylori* infection-associated pathologies, prevention should be the ultimate solution. Vaccines have been suggested as a cost-effective alternative to slow the emergence of drug resistance by decreasing infection rates and hence antibiotic usage [48]. However, an efficient vaccine has not yet been developed for several reasons. It is therefore important to perform susceptibility-guided

retreatment using a case-by-case approach, if available, in patients with initial treatment failure. Recently, a novel fully-automated rapid genetic analyzer was developed and shown to be capable of determining CAM resistance (e.g., 23S rRNA gene point mutations at A2143G and A2144G) within 60-120 min, compared to the 7-10 days required for culture testing [49]. Genotypic resistance testing is more convenient and rapid than standard culture susceptibility testing and has shown promising eradication results in Taiwan, with the potential to determine resistance even from stool samples [50]. In the near future, follow-up of eradication failure with genotypic resistance-guided methods could even offer tailored therapy for treatment of naïve patients.

The high prevalence of CAM, MNZ and LVX in this study was in agreement with the result of a previous study that a cure rate was only 69% when using 14 days course of MNZ- and LVX-based triple therapy [51]. Other alternative strategies such as bismuth or non-bismuth-based quadruple regimens or sequential therapy may be more effective in Bangladesh (Table 5) [7]. In fact, bismuth-based quadruple regimens with furazolidone and AMX or furazolidone-base triple regimens have shown sufficient efficacy in Bangladesh, with eradication rates of 100% and 96%, respectively [52]. However, this clinical trial was performed around 15 years ago. The current effectiveness of these therapies has not yet been established. Additional clinical trials are required to improve the rate of successful eradication in Bangladesh.

Table 5. Regions with reported resistance and potential rescue regimens for *H. pylori* eradication in Asia.

Resistance type	Country	First- and second-line therapy					Rescue therapy		
		CAM-based triple therapy	MNZ-based triple therapy	BIS-based quadruple therapy	non-BIS quadruple 'concomitant' therapy	Sequential therapy	Hybrid therapy	LVX-based triple therapy	RIF-based triple therapy
Low resistance to four antibiotics	Taiwan, Thailand, Malaysia	√	√	√	√	√	√	√	√
High CAM resistance (>20%)	Japan		√	√	√	√	√	√	√
High MNZ resistance (>40%)	China-Hong Kong, Saudi Arabia, Singapore, Bhutan	√		√	√	√	√	√	√
High CAM and MNZ resistance	Turkey, Bahrain			√	√		√	√	√
High CAM and LVX resistance	South Korea		√	√	√	√	√		√
High CAM, MNZ, and LVX resistance	China-Beijing and Southeast China, Vietnam, Bangladesh			√	√				√
High CAM, MNZ, and AMX resistance	Indonesia			√			√	√	√
High CAM, MNZ, AMX, and LVX (CIP) resistance	Iran, India, Pakistan			√					√

Abbreviations: CAM, clarithromycin; MNZ, metronidazole; LVX, levofloxacin; AMX, amoxicillin; CIP, ciprofloxacin; TCN, tetracycline.

Conclusions

The rates of resistance to CAM, MNZ, and LVX were high in Bangladesh, which suggests that CAM-, MNZ-, and LVX-based triple therapies are not useful as first-line therapies in Bangladesh. TCN can be still used, although domestic data regarding its successful eradication rate is lacking. National epidemiological surveillance of resistance rates is required in order to determine the optimal treatment strategies in Bangladesh.

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Authors' contributions

YY and HA designed the study; YY, HA and MM performed data analysis, data interpretation, and wrote the manuscript. PS, FA, AK contributed to data acquisition. YY revised the manuscript to include important content. All authors read and approved the final version of the manuscript.

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Corresponding author

Yoshio Yamaoka MD, PhD

Department of Environmental and Preventive Medicine, Oita University Faculty of Medicine, 1-1 Idaigaoka, Hasama-machi, Yufu-City, Oita 879-5593, Japan

Phone: +81-97-586-5740

Fax: +81-97-586-5749

Email: yyamaoka@oita-u.ac.jp

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