Effective Therapeutic Regimens in Two South Asian Countries with High Resistance of Major Helicobacter pylori Antibiotics

by Muhammad Miftahussurur

1 **Effective Therapeutic Regimens in Two South Asian Countries with High Resistance of**

2 **Major *Helicobacter pylori* Antibiotics**

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4 Short title: Effective *H. pylori* regiments in South Asia

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ABSTRACT

BACKGROUND

Nepal and Bangladesh occupied a high prevalence of *Helicobacter pylori* with the high resistance rates to clarithromycin, metronidazole, and levofloxacin. We evaluated susceptibility and genetic mutations of the five alternative antibiotics against isolates of both countries to obtain effective *H. pylori* regiments.

MATERIALS AND METHOD

Agar dilution method was used to determine the minimal inhibitory concentration of 42 Nepal and 56 Bangladesh strains and whole genome sequencing method was used for the mutation analysis.

RESULTS

The results demonstrated zero resistance to furazolidone and rifabutin and high susceptibility of sitafloxacin (95.2% in Nepal and 98.2% in Bangladesh). However, we observed high resistance to rifaximin (52.4% of Nepal and 64.3% of Bangladesh) as well as garenoxacin. But garenoxacin resistance was higher in Bangladesh than Nepal (51.6% vs. 28.6%, $P = 0.041$, respectively) and the poor activity of garenoxacin possibly due to its correlation with levofloxacin resistance ($P = 0.03$). Rifaximin and garenoxacin were frequently detected as double resistance (19.0% in Nepal and 41.0% in Bangladesh) with a significant correlation ($P = 0.014$), and all sitafloxacin resistance strains detected as triple resistance along with garenoxacin and rifaximin. The *gyrA* mutations may play important role in garenoxacin resistance and double mutations of A87 and D91 were associated with sitafloxacin resistance. The *rpoB* analysis demonstrated the known mutation; V657I in three strains and several novel mutations including V2592L, T2537A, and F2538L.

CONCLUSION

1 Rifabutin can be applied as therapy cautiously due to its interaction with tuberculosis endemic in

2 Bangladesh. High susceptibility of furazolidone as well as sitafloxacin, suggesting possible

3 future application in Nepal and Bangladesh.

4 **Keywords:** Nepal, Bangladesh, drug resistance, *Helicobacter pylori*, antibiotics

1 INTRODUCTION

Helicobacter pylori chronically occupies 50% of the human population worldwide and still become a major worldwide problem due to their role in the peptic ulcer diseases and gastric cancer pathogenesis [1]. South Asia is the most densely populated geographical region in the world with total citizens of 1,891,454,121 in 2017, most of the prevalence of *H. pylori* in several countries in this region is more than 50%, for examples, in Bangladesh the prevalence was reported to be 60.2% with a high re-infection rate [2, 3], and the prevalence was reported to be much higher in Bhutan to be 73.4% [4]. The high prevalence of *H. pylori* in this region was associated with gastroduodenal diseases especially peptic ulcer diseases, thus a high cost of eradication therapy is a factor should be considered. An important aspect to consider is the strategy to achieve successful therapy, not only to eradicate *H. pylori* infection but also improve clinical symptoms in gastroduodenal disease and halt the progression to gastric cancer, subsequently [5, 6]. However, the goal to achieve successful therapy was greatly challenged by the increase of antibiotic resistance rates in South Asian countries, primarily in first- and a second-line antibiotic to combat *H. pylori* infection such as clarithromycin, metronidazole, and levofloxacin [7].

Emerging *H. pylori* resistance to major antibiotics such as clarithromycin and metronidazole were reported. In addition, increasing resistance of levofloxacin, which has recently still been used in second-line regimens and as a rescue treatment for *H. pylori* eradication in South Asia was also observed. Previously, we reported a high resistance rate to clarithromycin and metronidazole (39.3% and 94.6%, respectively) in Bangladesh including an emerging antimicrobial resistance of levofloxacin (66.1%), but low amoxicillin and tetracycline resistance rates [7]. We also revealed a high metronidazole and clarithromycin resistance rate (88.1% and 21.4%, respectively) in Nepalese strains [8]. Importantly, both countries showed an overabundance of the breaking points required by the Maastricht guidelines on *H. pylori* infection management (20% for clarithromycin and 40% for metronidazole) [9] with high levofloxacin resistance, suggesting clarithromycin or metronidazole-based regimens and

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levofloxacin are insufficient as the first and second-line treatments for *H. pylori* eradication. Then, giving a reason why the *H. pylori* cure rate in South Asia region is around 70% [10], which is lower than the recommended eradication target which was 90-95% to be used [11].

Furazolidone is mentioned in the Maastricht Consensus V as an alternative drug [9] due to its efficacy, low rate of primary bacterial resistance and low-cost therapies [12]. Rifabutin, an anti-tuberculous agent which inhibits the transcription in *H. pylori* [13-15] may become alternative regiment and the resistance is thought to be associated with the *rpoB* mutation [16]. Rifaximin, a rifamycin group as same as rifabutin, had a little data but promising as *H. pylori* drug due to a poor absorbance to the blood with a minimal adverse effect and the high bioavailability in the gastrointestinal tract is also recommended [17] [9]. Other regimens such as garenoxacin and sitafloxacin, the novel quinolones are also mentioned to be effective for strains that have mutations in *gyrA* and *gyrB* [18].

Bangladesh and Nepal are two important countries that could be used as a model for a population with high clarithromycin and metronidazole resistance. The resistance data from both countries will give information for the alternative regiments can be proposed worldwide. Further, an investigation about an alternative regiment is undeniably required to decide the best regiment. The evaluation of the antibiotic susceptibility tests and molecular analysis is important to understand the resistance pattern and mechanism [19]. The aim of the present study was to provide the data regarding optional antibiotic with low resistance rate or the resistance is uncommon. In addition, we also analyzed the DNA sequences to find mutation candidates that might play a role in the antibiotic resistance.

MATERIAL AND METHOD

Patient Sampling and *H. pylori*

We used the same strains from previous studies [7, 8, 20]. The study was conducted in Nepal and Bangladesh which took the endoscopic biopsy samples of adult dyspeptic patients from

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Gastroenterology Department, Tribhuvan University Teaching Hospital (TUTH), Kathmandu, Nepal and Dhaka Medical College, Dhaka, Bangladesh [7, 8, 20]. As we mentioned in our previous studies, among 146 subjects from Nepal and 133 patients from Bangladesh, 42 strains from Nepal (35 gastritis, 4 duodenal ulcers, and 3 gastric cancer) and 56 strains from Bangladesh (53 gastritis and 3 peptic ulcer) were succeeded to isolate from homogenized antral biopsy specimens by inoculating into a selective agar plate and incubated up to 10 days in microaerophilic condition (10% O_2, 5% CO_2, and 85% N_2) at 37°C, and subsequently sub-cultured into antibiotic-free Mueller-Hinton II agar medium (Beckton Dickinson, NJ, USA) with 10% horse blood supplementation in the same microaerophilic conditions [7, 8]. *H. pylori* stock was stored in Brucella Broth (Difco, NJ, USA) with 10% dimethyl sulfoxide and 10% horse serum at -80°C. Written informed consent was obtained from all participants, and the protocol was approved by the Review Board or the Ethics Committee of Bangladesh Medical Research Council (BMRC), Dhaka, Bangladesh; TUTH, Kathmandu, Nepal and Oita University Faculty of Medicine, Yufu, Japan.

Antibiotic Susceptibility Test

Determination of susceptibility utilized the two-fold Agar Dilution Method based on the guideline from EUCAST [21]. Before performing antibiotic susceptibility test, we subcultured two times on the Brucella Agar Plate supplemented with the 7% horse blood for three days. We prepared Mueller Hinton agar supplemented with 10% horse blood with a different concentration of antibiotics. Bacterial suspension that collected from the agar plate was adjusted into OD 0.1 and inoculated into the blood agar plate that contained serial dilution of antibiotics. We examined five antibiotics including furazolidone (Tokyo Chemical Company, Tokyo, Japan) and rifabutin (Sigma Aldrich, St. Louis, MO, US) ranged from 0.063 to 8 µg/mL; rifaximin (Tokyo Chemical Company), garenoxacin (Sigma Aldrich) and sitafloxacin (Haoyuan chemexpress, Shanghai, China) from 0.063 to 32 µg/mL. The results were evaluated after 3-5 days incubation in the microaerophilic environment. A strain was determined as a resistant strain if the MIC

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exceeds the clinical breakpoints; >4 mg/L for furazolidone and rifaximin, >1 mg/L for rifabutin, garenoxacin, and sitafloxacin as previously describes [22-25]. We also determined the MIC50 as the same value of median and MIC90 as the same value of 90 percentile to represent the ability of the antibiotic to inhibit ≥50% and ≥90% of the all strains, respectively.

H. pylori **Mutation Analysis**

DNA extraction of cultured *H. pylori* was performed by using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's recommendation. The information for *gyrA* and *gyrB* mutation from Nepalese strains was available from our previous publication [8]. To complete the data, we obtained the *gyrA*, *gyrB* and *rpoB* sequences from Bangladeshi strains from our next generation sequencing (NGS) data (MiSeq next-generation sequencer; Illumina, Inc., San Diego, CA) utilizing BLAST algorithm implemented on the CLC Genomic Workbench software (ver. 11; Qiagen, Venlo, Netherlands). The queries for BLAST analysis were hp0701, hp0501 and hp1198 from *H. pylori* 26695 (GenBank accession number AE000511.1) for *gyrA*, *gyrB*, and *rpoB*, respectively. Briefly, after obtaining the sequence data, we confirmed there was neither insertion nor deletion leads to a frameshift mutation. Subsequently, we aligned based on the amino acid sequence using MAFFT (http://mafft.cbrc.jp/alignment/server/). For mutation related to rifaximin resistance, we compared all amino acids of resistant and the sensitive strains to the reference sequence using our original PERL script and confirmed by visual inspection. Variants found in both the resistant and the sensitive strains were considered as normal variants and were excluded from further analysis. Variants found in the resistant strains but not in the sensitive ones were considered as variants related to antibiotic resistance. For the mutation related to quinolone resistance, we compared the amino acids of all the resistant strains to strain 26695 and searched whether the presence of variant at the position 87 and 91 of *gyrA* and the position 481 and 484 of *gyrB*.

Statistical Analysis

1 We examined the effect of different variables on the strains susceptibility using multivariate

2 analysis and obtained odds ratios (OR) with 95% confidence intervals (CI). The variables

3 investigated in the binary logistic regression analysis included sex, age, and clinical outcomes. A

4 P-value <0.05 was considered statistically significant. The trend of age factor to the

5 susceptibility and analysis of susceptibility difference among the country were measured by

6 Linear Chi-square test. SPSS statistical software package version 23.0 (SPSS, Inc., Chicago, IL)

7 was used for all statistical analyses.

1 **RESULT**

2 **Five Antibiotic Resistance Rates**

3 Table 1 shows the summary of the antibiotic resistance of *H. pylori* isolated in Nepal and

4 Bangladesh. Agar dilution method revealed that there was no resistant strain observed towards

5 furazolidone and rifabutin among 42 Nepalese strains and 56 Bangladesh strains. Sitafloxacin

6 could become a prospective alternative regiment due to a low resistance rate (2/42, 4.8% in

7 Nepal and 4.8% in Bangladesh). In contrast, we observed high resistance of garenoxacin

8 reaching 51.8% (29/56) in Bangladesh which is higher than in Nepal 28.6% (12/42, P = 0.041).

9 Among the quinolone group, garenoxacin had a higher resistance than sitafloxacin in Bangladesh

10 or in both countries (P = 0.015 and P = 0.005).

11   Rifaximin resistance in both countries were also high (64.3%, 36/56 vs. 52.4%, 22/42, P =

12 0.79). The double resistance, especially garenoxacin and rifaximin resistant which appeared

13 together in one strain, occurred frequently in Bangladesh and Nepal (41.1% and 19.0%).

14 Interestingly, we found an association between garenoxacin and rifaximin resistance in

15 Bangladesh strains (P = 0.014), but not in Nepal strains (P = 0.32). We also observed triple

16 antibiotic resistance with the highest prevalence was the combination of levofloxacin,

17 metronidazole, and rifaximin in both countries (7/98, 7.1%) (Supplement Table 1). Furthermore,

18 higher quadruple, quintuple, and sextuple resistance in combination with the clarithromycin,

19 amoxicillin, metronidazole, levofloxacin, were also observed in Bangladesh compared to Nepal

20 (Supplement Table 1). Quintuple resistance in Bangladesh was reaching 23.2% and the highest

21 quadruple resistance was 14.3% among the population.

22   The distribution of resistance to garenoxacin and rifaximin between male and female was

23 close to equal either in both or each country (P >0.05, Table 2). However, we observed the

24 resistance of rifaximin might associate with age as we found increasing rate in the 30-39 years

25 old (P = 0.047, OR = 15.0, CI 95% 1.030-218.300) and 40-49 years old age group (P = 0.03, OR

26 = 16.0, CI 95% 1.315-194.623) in Nepal. The calculation in both population of two countries

27 also showed an association of occurrence of rifaximin resistance in the age group 40-49 years old

1 (P = 0.034, OR = 6.4, CI 95% 1.156-35.437). The association of resistance with age also showed

2 in garenoxacin. The highest garenoxacin resistance rate was observed in age with less than 30

3 years old age group. In both populations, it also showed a trend that the occurrence of resistance

4 was increasing to the younger age (P = 0.051).

5

Table 1. Antibiotic susceptibility of *H. pylori* isolates from Nepal and Bangladesh

Antibiotics (%)	Country (n)		
	Nepal (42)	Bangladesh (56)	Both Countries (98)
Rifaximin	22/42 (52.4)	36/56 (64.3)*	58/98 (59.2)
Furazolidone	0/42 (0.0)	0/56 (0.0)	0/98 (0.0)
Rifabutin	0/42 (0.0)	0/56 (0.0)	0/98 (0.0)
Garenoxacin	12/42 (28.6)	29/56 (51.8)**	41/98 (41.8)
Sitafloxacin	2/42 (4.8)	1/56 (1.8)	3/98 (3.1)

6 * P value Rifaximin 0.789

7 ** P value Garenoxacin 0.041

8

9 Table 2. Distribution of antibiotic resistance in Nepal and Bangladesh patients

Characteristic	n	Resistant Regiment (%)				
		Rifaximin	Furazolidone	Rifabutin	Garenoxacin	Sitafloxacin
Both Countries						
Sex (%)						
Male	42	24/42 (57.1)	0/42 (0.0)	0/42 (0.0)	20/42 (47.6)	0/42 (0.0)
Female	56	34/56 (60.7)	0/56 (0.0)	0/56 (0.0)	21/56 (37.5)	3/56 (5.4)
Age (%)						
<30	27	15/27 (55.6)	0/27 (0.0)	0/27 (0.0)	15/27 (55.5)	0/27 (0.0)
30-39	26	15/26 (57.7)	0/26 (0.0)	0/26 (0.0)	12/26 (46.1)	1/26 (3.8)
40-49	21	16/21 (76.2)	0/21 (0.0)	0/21 (0.0)	7/21 (33.3)	1/21 (4.8)
50-59	15	9/15 (60.0)	0/15 (0.0)	0/15 (0.0)	4/15 (26.7)	1/15 (6.7)
>59	9	9/15 (60.0)	0/15 (0.0)	0/15 (0.0)	4/15 (26.7)	0/15 (0.0)
Clinical Outcome (%)						

11

Gastritis	88	53/88 (60.22)	0/88(0.0)	0/88(00)	40/88(45.5)	3(3.5)
Duodenal Ulcer	4	2/4(50)	0/4(0.0)	0/4(0.0)	0/4(0.0)	0(0.0)
Peptic Ulcer	3	2/3 (66.7)	0/3(0.0	0/3(0.0	1/3(0.0)	0(0.0)
Gastric cancer	3	1/3(33.3)	0/3(0.0)	0/3(0.0)	0/3(0.0)	0(0.0)

Nepal
Sex (%)

Male	16	8/16 (50.0)	0/16 (0.0)	0/16 (0.0)	5/16 (31.3)	0/16 (0.0)
Female	26	14/26 (53.8)	0/26 (0.0)	0/26 (0.0)	7/26 (26.9)	2/26 (7.7)

Age (%)

<30	10	4/10 (40.0)	0/10 (0.0)	0/10 (0.0)	4/10 (40.0)	0/10 (0.0)
30-39	7	5/7 (71.4)*	0/7 (0.0)	0/7 (0.0)	2/7 (28.6)	0/7 (0.0)
40-49	11	8/11 (72.7)*	0/11 (0.0)	0/11 (0.0)	3/11 (27.3)	1/11 (9.1)
50-59	7	4/7 (57.1)	0/7 (0.0)	0/7 (0.0)	2/7 (28.6)	1/7 (14.3)
>59	7	1/7 (14.3)	0/7 (0.0)	0/7 (0.0)	1/7 (14.3)	0/7 (0.0)

Clinical Outcome (%)

Gastritis	35	19/35(54.3)	0/35(0.0)	0/35(0.0)	12/25(34.3)	2/35(5.7)
Duodenal Ulcer	4	2/4(50.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)
PUD	0	0/0 (0.0)	0/0 (0.0)	0/0 (0.0)	0/0 (0.0)	0/0 (0.0)
Gastric cancer	3	1/3(33.3)	0/3(0.0)	0/3(0.0)	0/3(0.0)	0/3(0.0)

Bangladesh
Sex (%)

Male	26	16/26 (61.5)	0/26 (0.0)	0/26 (0.0)	15/26 (57.7)	0/26 (0.0)
Female	30	20/30 (66.7)	0/30 (0.0)	0/30 (0.0)	14/30 (46.7)	1/30 (3.3)

Age (%)

<30	17	11/17 (64.7)	0/17 (0.0)	0/17 (0.0)	11/17 (64.7)	0/17 (0.0)
30-39	19	10/19 (52.6)	0/19 (0.0)	0/19 (0.0)	10/19 (52.6)	1/19 (5.3)
40-49	10	8/10 (80.0)	0/10 (0.0)	0/10 (0.0)	4/10 (40.0)	0/10 (0.0)
50-59	8	5/8 (62.5)	0/8 (0.0)	0/8 (0.0)	2/8 (25.0)	0/8 (0.0)
>59	2	2/2 (100)	0/2 (0.0)	0/2 (0.0)	2/2 (100)	0/2 (0.0)

Clinical Outcome (%)

Gastritis	53	34/53 (64.2)	0/53 (0.0)	0/53 (0.0)	28/53 (52.8)	1/53 (1.9)

Duodenal Ulcer	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
PUD	3	2/3 (66.7)	0/3/ (0.0)	0/3 (0.0)	1/3 (33.3)	0/3 (0.0)
Gastric cancer	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

1   *P = 0.03, OR=16, CI 95% 1.315-194.623

2   ** P = 0.047, OR=15, CI 95%= 1.030-218.300

3

4   **Comparison with Standard Antibiotics**

5   We compared our results with the susceptibility data of standard antibiotics from our previous

6   studies in the same strains [7, 26]. We confirmed that high resistance of clarithromycin and

7   metronidazole were prominent characteristic in Nepal and Bangladesh population. Furazolidone

8   and rifabutin appeared to be a potential and effective regiment to eradicate clarithromycin- and

9   levofloxacin-resistant *H. pylori* infection, as shown by their zero-resistance rate (Table 3).

10 Promising results were also showed in sitafloxacin regimen to overcome the high rate of

11 levofloxacin resistance although still had some resistant strain,4.8% (2/42) in Nepal and 1.8%

12 (1/56) in Bangladesh. In contrast, we found an association between the resistance to garenoxacin

13 and levofloxacin in Nepalese (P = 0.003). Furthermore, all garenoxacin resistant strains were

14 resistant to levofloxacin in Bangladesh. Thus, sitafloxacin application may be superior compared

15 to garenoxacin in both countries.

16   Interestingly, although sitafloxacin was superior to levofloxacin, the association between

17 clarithromycin and sitafloxacin resistance in Nepalese strains was significant (P = 0.042). All of

18 two sitafloxacin resistant strains also had clarithromycin resistance. In Bangladesh, we found the

19 association between sitafloxacin and amoxicillin resistance (P = 0.036) although both of them

20 possessed low resistance rate. All sitafloxacin resistance occurred as triple resistance together

21 with garenoxacin and rifaximin, and those strains also showed resistant to metronidazole,

22 clarithromycin, and levofloxacin. When we analyzed data based on clarithromycin resistance,

23 garenoxacin which exhibited a quite high resistance rate was associated with clarithromycin

24 resistance, especially in Nepalese strains (P = 0.015). Meanwhile, rifaximin resistance in this

1 study may not affect clarithromycin and levofloxacin resistance which suggest a specialized

2 mechanism of resistance.

3

4 Table 3. Comparison with previous results of different antibiotic susceptibility test

Resistant Regiment (%)	Country	
	Nepal (42)*	Bangladesh (56)*
Clarithromycin	9/42 (21.4)	22/56 (39.3)
Amoxicillin	0/42 (0.0)	2/56 (3.6)
Metronidazole	37/42 (88.1)	53/56 (94.6)
Tetracycline	0/42 (0.0)	0/56 (0.0)
Levofloxacin	18/46 (42.9)	37/56 (66.1)
Garenoxacin	12/42 (28.6)	29/56 (51.8)
Sitafloxacin	2/42 (4.8)	1/56 (1.8)
Furazolidone	0/42 (0.0)	0/56 (0.0)
Rifabutin	0/42 (0.0)	0/56 (0.0)
Rifaximin	22/42 (52.4)	36/56 (64.3)

5

6 **Distribution of MIC**

7 The range of MIC from each antibiotic was varied. Interestingly, both countries showed a similar

8 pattern of bimodal (Figure 1), as a sign of a primary resistance and associated with mutation role,

9 but not secondary or innate resistance [27]. Rifabutin, which is very susceptible to all strains in

10 both countries exhibited very low minimum inhibitory concentration under 0.063 µg/mL. In

11 furazolidone, most of the MIC was observed in 0.25 µg /mL which were sensitive. Sitafloxacin

12 MIC was ranged from 0.063 to 2 µg/mL, but the majority was in the 0.063 µg/mL concentration

13 which was sensitive. However, a wide range of MIC observed rifaximin, from 1 to 16 µg/mL, as

14 well as in garenoxacin ranged from 0.063 to 8 µg/mL.

15

16 **Figure legend**

17 **Figure 1. Minimal Inhibitory Concentration Distribution of Nepal and Bangladesh Strains.**

18 Although the MIC range from each antibiotic was varied, a bimodal pattern was shown in

1   Rifaximin and Garenoxacin, suggesting as a primary resistance and associated with the genetic

2   mutation.

3

4   Among the rifampin group, MIC90 measurement which means the MIC an antibiotic could

5   eliminate 90% of the isolates, demonstrated that rifabutin was 256 times superior to rifaximin in

6   Bangladesh and Nepal strains. Furazolidone also showed relatively low MIC90, which was in

7   0.25 µg/mL. Even though previously reported that Nepal and Bangladesh strains had a high

8   resistance of levofloxacin, our current results showed that MIC90 of garenoxacin achieved at the

9   concentration 2 µg/mL, 8 times lower than rifaximin. Sitafloxacin also demonstrated MIC90 4

10   times lower than garenoxacin.

11

12   **Molecular Detection**

13   Resistance in the rifampin group, including rifaximin and rifabutin, were related to the mutation

14   on the RNA Polymerase gene or *rpoB* [28]. In this study, we extracted the *rpoB* gene and

15   randomly picked 5 susceptible strains and compared with all rifaximin resistant strains (Table

16   Suppl. 2). As shown in the previous study which detected several mutations in *rpoB* associated

17   with rifamycin [28], we found that Isoleucine replaced Valine amino acid in the position 657 in

18   only 3 strains. However, to our knowledge, there was no study reported the mutation related to

19   the rifaximin resistance. We confirmed that several mutation loci such as I2619V which

20   appeared in 43.5% resistance strains (Table 4). Other mutations such as V2592L, T2537A, and

21   F2538L might also the candidate mutations which related to the rifaximin resistance.

22   In this study, we analyzed the presence of a mutation in the *gyrA* and *gyrB* within

23   fluoroquinolone drug-resistance strains, as shown in Table 5. We used the data obtained from

24   next-generation sequencing to analyze the mutation in Bangladesh strains. In Nepal, we used our

25   previous study published data [8]. Table 5 shows the mutation found the *gyrA and gyrB* in

26   Bangladesh and Nepal strains. In Bangladesh strain, single mutation either in position 87 or 91

27   belonged to 79.3% (23/29) of garenoxacin resistance strain. The most common mutation is

15

1   N87K which detected in 31.0% (9/29), followed by D91N 27.6% (8/29) and D91G which were

2   present in 17.2% (5/29). We found that 5 strains (17.2%) did not possess a mutation in *gyrA*,

3   suggesting another mechanism of resistant. There was 1 strain possessed double mutation at

4   N87K and D91N and showed resistant to both garenoxacin and sitafloxacin with considerably

5   high MIC (64 µg/mL for garenoxacin and 2 µg/mL for sitafloxacin). In Nepal, 80% (8/10) of the

6   garenoxacin resistant strain had a single mutation in either position 87 or 91. We also observed

7   in sitafloxacin resistant strain that 10% (1/10%) had a double mutation in N87K and D91N.

8   Another sitafloxacin resistant strain also had a double mutation in D91N and R130K. Thus, a

9   mutation at the position R130K that might become a new candidate for sitafloxacin resistance.

10   We evaluated the mutation of gyrB which consisted of substitution in D481E and R484K.

11   Glutamic acid replaced aspartic acid at the position 481 and Lysine replaced Arginine at 484 of

12   the *gyrB* were detected in 55.2% (16/29) of Bangladeshi strains (Table 6), as previously

13   described [29]. Interestingly, most of the garenoxacin-resistant strains possessing double

14   substitution in D481E and R484K (14/29; 48.3%) and a single mutation in D481E was 3.4%

15   (1/29) or R484K only appeared in 3.4% (1/29). In Nepal strain, an only E483K mutation in one

16   resistant strain was observed.

17

Table 4. Mutation frequency of *rpoB* gene

No	Mutation Locus	Frequency (%)
1	I2619V	17 (43.59)
2	V2592L	14(35.9)
3	T2537A	14(35.9)
4	F2538L	14(35.9)
5	K2359S	13 (33.3)
6	K2594R	14 (33.3)
7	D2381E	11 (28.21)
8	T1540A	7 (17.9)
9	E2809D	8 (17.9)
10	N2603D	9 (17.9)
11	P1965T	10 (17.9)
12	K231E	5 (12.8)
13	N1599S	6 (12.8)

18

Table 5. Mutation in *gyrA* (87 and 91 positions) and *gyrB* (481, 483, 484 positions) in garenoxacin and sitafloxacin resistance

gyrA mutation data

Mutation	Frequency (%)	MIC LEV	MIC GAR	MIC SIT
Bangladesh				
N87K	9/29 (31.0)	4-16	1-2	0.063-0.5
D91G	5/29 (17.3)	2-4	1-2	0.063-0.25
D91N	8/29 (27.6)	2-64	1-8	0.063-0.25
D91Y	1/29 (3.4)	4	2	0.125
N87K, D91N	1/29 (3.4)	64	64	2
No mutation	5 (17.3)	4-16	1-2	0.063-0.25
Nepal				
N87K	3/10 (30.0)	32	1-2	0.5
N87I	1/10 (10.0)	32	4	0.5
D91N	2/10 (20.0)	32	2	0.125-1
D91Y	1/10 (10.0)	32	2	0.25
N87K, D91N	1/10 (10.0)	32	4	2
D91N, R130K	1/10 (10.0)	32	2	1
No mutation	1/10 (10)	32	1	0.5

gyrB mutation data

Mutation	Frequency (%)	MIC LEV	MIC GAR	MIC SIT
Bangladesh				
D481E	1/29 (3.5)	2	2	0.25
R484K	1/29 (3.4)	64	8	4
D481E, R484K	14/29 (48.3)	2-64	1-8	0.063-0.5
No mutation	13/29 (44.9)	4-16	1-2	0.063-0.5
Nepal				
E483K	1/10 (10.0)	32	4	0.5
No mutation	9/10 (90.0)	32	1-4	1-2

MIC: Minimum Inhibitory Concentration, µg/mL

LEV: Levofloxacin GAR: Garenoxacin; SIT: Sitafloxacin

DISCUSSION

We evaluated five antibiotics as an attempt to find effective regiment in the region with high resistance of clarithromycin, metronidazole, and levofloxacin such as in Nepal and Bangladesh. We revealed that the rifabutin and furazolidone were susceptible to all strains including clarithromycin, levofloxacin, and metronidazole-resistant strains with a very low MIC. Our result was concordance with several studies, which also demonstrated a very low resistance rate in vitro and could achieve a 96.6% cure rate in the clinical trial [30-32]. Thus it becomes a recommended drug for the rescue therapy in the region with high quinolone resistance rate as mentioned on the Maastricht V [9]. However, some cautions should be applied due to its interaction with tuberculosis which is prevalent in South Asia as it contributed to 40% of cases of the world tuberculosis [33]. In addition, the interaction with Cytochrome P450 may induce cross-reaction with other drugs and severe adverse effect such as myelotoxicity are the limitation of rifabutin, though some cases resolved by observation [34].

Furazolidone also showed promising results for both countries by its zero-resistance rate. The possible future application of this regimen in Nepal and Bangladesh was supported by several studies which proposed furazolidone as the initial therapy regiment [35, 36] because it could reach more than 90% cure rate in areas with high clarithromycin, metronidazole or levofloxacin resistance, such as China and Iran [37, 38]. The cost was also relatively lower compared to other drugs such as sitafloxacin, thus more suitable in developing countries [39]. Even though furazolidone was mentioned to have a carcinogenic effect, the IARC reported that this regiment was included in Class3, means "unclassifiable" as to carcinogenicity in human. This classification was less harmful than metronidazole which listed as Class 2B, means "definite" carcinogen in animals and in humans [20]. In fact, the furazolidone related side effect was also reported to be relatively low [40].

Quinolone resistance is also a major problem in *H. pylori* eradication, not only in Nepal and Bangladesh but also worldwide. To obtain a better understanding, we compared the levofloxacin, garenoxacin and sitafloxacin susceptibility and the mutation pattern in *gyrA* and *gyrB*

18

from the same strains. Sitafloxacin showed stronger elimination capacity than garenoxacin and levofloxacin. This result was supported by several previous studies from Japan which proposed sitafloxacin to overcome levofloxacin resistance which was caused by a mutation in *gyrA* [25, 41, 42]. Therefore, sitafloxacin might be ideal to treat even the strain that has a mutation in *gyrA* in the other population than Japan, even though the availability outside Japan is limited.

However, garenoxacin susceptibility was low and this poor activity also supported by our finding that garenoxacin resistance was related to the levofloxacin. Although garenoxacin is safe and does not have any significant harmful side effects in the oral consumption [43], it may not suitable in Nepal and Bangladesh because the susceptibility was less than 90% as suggested by the Maastricht consensus [9]. In contrast with the previous study that mentioned the resistant was low at a young age less than 30 years old [44], we found the resistance tended to increase by the younger age in Nepalese. Increasing usage of quinolones such as levofloxacin in the early ages related to the poor susceptibility of garenoxacin [45], many antibiotic prescriptions at the same times and lack of education of usage were reported in Nepal [46]. Our results suggest that the importance of quinolone utilization regulation is necessary for South Asia.

In concordant with the previous study, molecular analysis for the *gyrA* and *gyrB* in the present study also showed that single mutation in one of position 87 and 91 related to resistance phenotype in levofloxacin and garenoxacin [47], while double mutations (position at 87 and 91) related to sitafloxacin resistance [48]. Our study also found the possible co-resistance phenomena between quinolones such as sitafloxacin and garenoxacin with amoxicillin, metronidazole, clarithromycin, and rifaximin resistances may indicate other mechanisms such as altered drug permeation [49] or increase expression of efflux pumps which reported to be responsible for multidrug resistance [50].

In the present study, we observed that the primary resistance of rifaximin was high in both countries. In addition, it demonstrated higher MIC90 than several previous studies [51, 52]. We should take a note that poor activity of rifaximin was probably due to the application as a single regiment; however, triple therapy of rifaximin with proton pump inhibitor and levofloxacin or

clarithromycin in clinical studies also determined a poor cure rate with ranged from 30-58% [53]. Importantly, rifaximin had high safety because it is not absorbed well in the stomach, thus it barely approaches *H. pylori* which live under the mucus and epithelium [54]. However, this safety feature may useful for infection in children as described by a clinical trial in Russian children with 85.4% cure rate [55]. We analyzed the *rpoB* gene in rifaximin resistant strains but only three strains had a mutation at V657I as mentioned in previous studies [51, 56, 57]. We confirmed several novel mutations which appeared in almost half of Nepal and Bangladesh strains, suggesting other mutation or mechanism may give a higher influence rather than V657I.

The spreading of *H. pylori* strains contained resistance mutations may relate to the high double resistance in the garenoxacin and rifaximin [58]. Higher double resistance occurred higher in the Bangladeshi rather than Nepalese population can be associated with the fact that Bangladeshi density was 10 times higher than the Nepalese population thus facilitate the spread of resistance strain[7]. Accumulation of mutation and acquisition of resistance plasmid may also play role in triple resistance, quadruplet resistance and quintuplet resistance which occur in sitafloxacin resistance strain, thus affecting the target side of another antibiotic [59].

The main limitation of this study was the low number of the subject which participated in each country. Also, we only provided the in vitro result which may need further confirmation from in vivo study. Despite the limitations, this study has important finding and probably the first evaluation on the susceptibility of salvage therapy in Nepal and Bangladesh which is necessary for the future treatment of *H. pylori*. The availability of furazolidone and sitafloxacin in each country also become a disadvantage to choosing those antibiotics as the main therapy for *H. pylori*.

CONCLUSION

We confirmed that all strains from Nepal and Bangladesh are susceptible to furazolidone and rifabutin. In addition, very low resistance rate of sitafloxacin resistant strains in these countries, suggesting possible application in future to overcome the resistance of clarithromycin, metronidazole, and levofloxacin. The high resistance in garenoxacin was observed and may be

explained by the presence of *gyrA* and *gyrB* mutation. The mutation analysis of the *rpoB* also might explain high rifaximin resistance. Therefore, this data could give a prior information to choose the more suitable regiment.

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Authors' contributions

Conceived and designed the experiments: YY, MM, HA, and PKS. Performed the experiments: PS, MM, LAW, and DD. Analyzed the data: MM, YY, LAW, DD, and KAF. Contributed reagents/materials/analysis tools: RPS, FA, AAK. Wrote the paper: MM, YY, and KAF.

Disclosures

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1

Nucleotide Sequencing Data

These sequence data have been submitted to the DDBJ databases under accession number

'LC425712-LC425829' which can be accessed in http://www.ddbj.nig.ac.jp/.

Potential competing interests

The authors declare that they have no competing interests.

22

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