Emerging *Helicobacter pylori* levofloxacin resistance and novel genetic mutation in Nepal

Muhammad Miftahussurur1,3,4, Pradeep Krishna Shrestha2, Phawinee Subsomwong1, Rabi Prakash Sharma2 and Yoshio Yamaoka1,3*

**Abstract**

**Background:** The prevalence of *Helicobacter pylori* antibiotic susceptibility in the Nepalese strains is untracked. We determined the antibiotic susceptibility for *H. pylori* and analyzed the presence of genetic mutations associated with antibiotic resistance in Nepalese strains.

**Results:** This study included 146 consecutive patients who underwent gastroduodenal endoscopy in Kathmandu, Nepal. Among 42 isolated *H. pylori*, there was no resistance to amoxicillin and tetracycline. In contrast, similar with typical South Asian patterns; metronidazole resistance rate in Nepalese strains were extremely high (88.1 %, 37/42). Clarithromycin resistance rate in Nepalese strains were modestly high (21.4 %, 9/42). Most of metronidazole resistant strains had highly distributed *rdxA* and *frxA* mutations, but were relative coincidence without a synergistic effect to increase the minimum inhibitory concentration (MIC). Among strains with the high MIC, 63.6 % (7/11) were associated with frameshift mutation at position 18 of *frxA* with or without *rdxA* involvement. However, based on next generation sequencing data we found that one strain with the highest MIC value had a novel mutation in the form of amino acid substituted at Ala-212, Gln-382, Ile-485 of *dppA* and Leu-145, Thr-168, Glu-117, Val-121, Arg-221 in *dppF* aside from missense mutations in full-length *rdxA*. Mutations at Asn-87 and/or Asp-91 of the *gyrA* were predominantly in levofloxacin-resistant strains. The *gyrB* mutation had steady relationship with the *gyrA* 87–91 mutations. Although three (44.4 %) and two (22.2 %) of clarithromycin resistant strains had point mutation on A2143G and A2146G, we confirmed the involvement of *rpl22* and *infB* in high MIC strains without an 23SrRNA mutation.

**Conclusions:** The rates of resistance to clarithromycin, metronidazole and levofloxacin were high in Nepalese strains, indicating that these antibiotics-based triple therapies are not useful as first-line treatment in Nepal. Bismuth or non-bismuth-based quadruple regimens, furazolidone-based triple therapy or rifabutin-based triple therapy may become alternative strategy in Nepal.

**Keywords:** Nepal, Drug resistance, *Helicobacter pylori*, Genetic mutation

**Background**

The achievement of *Helicobacter pylori* against very hostile environment colonized on the stomach of over half of the world's population enact as the most successful human pathogens coexisted nearly sixty thousands years [1]. Although most of individuals exhibit overt disease leading to the hypothesis that the bacterium might be harmless and commensally, chronic infection of *H. pylori* represents a key factor in the etiology of various gastrointestinal diseases including chronic gastritis, peptic ulcer and mucosa-associated lymphoid tissue lymphoma. The outcome of each individual infection is capricious, similar to the rate of progression of the gastric mucosal damage. However, further progression is halted by eradication [2]. A recent meta-analysis supported that *H. pylori* eradication adequately decreases the rate of gastric malignancy, and the magnitude of the protective impact is more

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noteworthy among individuals with higher baseline gastric cancer risk [3]. Nevertheless, the adequacy of the standard first-line regimen containing a proton pump inhibitor, amoxicillin (AMX) and clarithromycin (CAM) or metronidazole (MNZ) has been seriously challenged and eradication rates below 70 % have been accounted in numerous countries, including South Asia [4, 5].

*H. pylori* antibiotic resistance mechanisms have been recognized in view of the different site-specific mutations that can be distinguished by molecular methods. It is important as a premise for consideration of more rational antibiotic combinations. One mechanism of CAM resistance has been elucidated due to one of five well-known point mutations (A2142G, A2143G, A2142C, A2144T, T2717C and C2694A) in the 23S rRNA [6, 7]. Our previous report demonstrated higher MICs associated with the synergic effect of mutated sequences in *infB* (hp0104), *rpl22* (hp1314) and A2143G [8]. Additionally, inactivation mutation including frameshift mutation, insertions and deletions of the *rdxA* (hp0954) and *frxA* (hp0642) [9]. Novel mutations including *rpsU* (hp0562) [10], *dppA* (hp0298), *dppB* (hp0299), *rpsA* (hp1294), *ackA* (hp0903), *rnc* (hp0662) and *dapF* (hp0566) were associated with MNZ resistance [11]. On the other hand, the mechanism of fluoroquinolone resistance in *H. pylori* has been identified to be linked to mutations in the quinolone resistance determining regions of the *gyrA* and *gyrB*, coding of the DNA gyrase [12]. Dual mutations in *gyrA* is accounted for a greater impact, while *gyrB* frequently occurred alongside *gyrA* mutations [13].

Nepal is a small landlocked country in South Asia with a low incidence of gastric cancer (5.3 cases per 100,000 populations per year; GLOBOCAN 2012; http://globocan.iarc.fr). Although it was varied between studies (16.3−70.5 %) [14−19], we confirmed the prevalence of *H. pylori* infection is 38.4 % (56/146) using several diagnostic test [20]. The majority of strains are so-called Western-type-*cagA* in Nepal as similar to typical South Asian patterns [20]. However, the mountainous people of northern Kathmandu are culturally linked to the Buddhists of Tibet, have higher prevalence of *H. pylori* infection and high-risk gastric mucosal atrophy than those Kathmandu people, the capital and the largest urban agglomerate of Nepal [21]. It is suggested lay stress on the need for *H. pylori* eradication in Nepal. Local antibiotic resistances screening are a key to counter primary *H. pylori* treatment failure, thus, reduce possibility spreading of secondary antibiotic resistance [4].

The prevalence of *H. pylori* antibiotic susceptibility in the Nepalese strains is untracked. Table 1 summarized *H. pylori* antibiotics resistance rates in South Asia. Generally, South Asian countries are the high CAM and MNZ resistance prevalence region [5]. Moreover, India and Bangladesh strains demonstrated emerging levofloxacin (LVX) resistance [22, 23], the second-line regimen drug and as a rescue treatment for *H. pylori* eradication. In recent years, antibiotic resistance is expanding overall [24, 25], it is critical to look at current drug resistance rates in Nepal. In this study, we aimed to determine the antibiotic susceptibility of *H. pylori* to CAM, MNZ, AMX, tetracycline (TCN), and LVX. Furthermore, we also determined the presence of genetic mutations associated antibiotic resistance in Nepalese strains.

**Methods**

**Patients and H. pylori**

This study included 146 consecutive patients (76 women and 70 men; mean age of 42.2 ± 15.7 years) consecutively from July 2012 to September 2012. The survey was conducted at the endoscopy services section of the Gastroenterology Department, Tribhuvan University Teaching Hospital (TUTH), Kathmandu, Nepal. Peptic ulcer diseases, including gastric and duodenal ulcers, were 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.

For *H. pylori* culture, antral biopsy specimens were homogenized and inoculated onto Mueller Hinton II Agar medium (Becton Dickinson, NJ, USA) supplemented with 7 % horse blood without antibiotics. 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 reactions for oxidase, catalase, and urease. Isolated strains were stored at −80 °C in Brucella Broth (Difco, NJ, USA) containing 10 % dimethyl sulfoxide and 10 % horse serum.

**Antibiotic susceptibility testing**

E-test (Biomerieux, Marcy l’Etoile, France) was used to determine the minimum inhibitory concentration (MIC) of AMX, MNZ, TCN, CAM, and LVX. Mueller-Hinton II Agar medium (Becton Dickinson) supplemented with 10 % defibrinated horse blood was used as culture media. The bacterial suspension, adjusted to be equivalent to a McFarland opacity standard of 3.0, was inoculated onto the plates. After 72 h 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

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**Table 1.** Summary of Antibiotic Resistance Rates in South Asia. Generally, South Asian countries are the high CAM and MNZ resistance prevalence region [5]. Moreover, India and Bangladesh strains demonstrated emerging levofloxacin (LVX) resistance [22, 23], the second-line regimen drug and as a rescue treatment for *H. pylori* eradication. In recent years, antibiotic resistance is expanding overall [24, 25], it is critical to look at current drug resistance rates in Nepal. In this study, we aimed to determine the antibiotic susceptibility of *H. pylori* to CAM, MNZ, AMX, tetracycline (TCN), and LVX. Furthermore, we also determined the presence of genetic mutations associated antibiotic resistance in Nepalese strains.

| Antibiotic | Resistance Rate (% of Samples) |
|------------|-------------------------------|
| AMX        | 90.7                          |
| MNZ        | 85.2                          |
| TCN        | 50.0                          |
| CAM        | 60.1                          |
| LVX        | 30.0                          |

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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.

Molecular detection on resistant strains

Mutations in gyrA, gyrB, rdxA, frxA and 23S rRNA were assessed on antibiotic-resistant strains by polymerase chain reaction (PCR) based sequencing. H. pylori DNA was extracted from H. pylori cultured to confluence on MNZ-resistant strains, gyrA and gyrB for LVX-resistant strains and 23S rRNA peptidyl transferase for CAM-resistant strains were amplified using the primers on the Additional file 1: Table S1 as described previously [13, 26, 27]. As a control, we sequenced randomly selected 4-sensitive MNZ and LVX strains and 2-sensitive CAM strains. The PCR products were analyzed by gel electrophoresis using 1.5% agarose gel sensitive CAM strains. The PCR products were amplified using the primers on the 23S rRNA, infB, rpl22 [8], rdxA, frxA, rpsL [10], dppA, dppB, rps4, ackA, rnc and dapF [11] from next-generation sequencing (NGS) data (MiSeq next-generation sequencer: Illumina, Inc., San Diego, CA). MiSeq output was integrated into contig sequences by CLC Genomics Workbench 7.0.4. Genomics Workbench was also used for gene prediction and translation to protein sequences.

Statistical analysis

Discrete variables were tested using the chi-square test, while continuous variables were tested using the Mann–Whitney U and t-tests. 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

Prevalence of antibiotic resistance

The prevalence of H. pylori infection was 37.7 % (55/146) based on histology confirmed by immunohistochemistry, whereas using culture it was 34.9 % (51/146) [20]. However, 9 isolates did not grow when subcultured onto Mueller Hinton II Agar medium from antibiotic selection plate. Finally, a total of 42 H. pylori strains were successfully isolated; consisting 16 male (age range, 17 to 77 years; mean age, 42.3 ± 18.9 years) and 26 female patients (age range, 17 to 69 years; mean age 43.3 ± 14.8 years). The patients consisted of 35 with chronic gastritis, 4 with peptic ulcer diseases and 3 with gastric cancer. Overall, only three strains showed sensitive to all antibiotics (7.14 %). Interestingly, there was no AMX- and TCN-resistant strains and these strains had low MIC predominant (90.5 % for 0.016 mg/L or less for AMX and for 0.25 mg/L or less for TCN, respectively) (Table 2). In contrast, similar with typical South Asian pattern [5]; MNZ resistance rate in Nepalese strains showed an emerging antimicrobial resistance pattern (88.1 %, 37/42) with MIC values 64 mg/L or more (26/37, 70.3 %, Fig. 1). In addition, although CAM resistance rate in Nepalese strains were modestly high (21.4 %, 9/42), we detected a high prevalence of LVX resistance (42.9 %, 18/42) with a high distribution of great MIC

Table 1  H. pylori antibiotics resistance rates in South Asia

| Ref  | Country   | City           | Year    | Patients | Methods | CAM   | MNZ   | LVX   | TCN   | AMX   | Others                  |
|------|-----------|----------------|---------|----------|---------|-------|-------|-------|-------|-------|-------------------------|
| [22] | India     | Gujarat        | 2008–2011| 80       | DDM     | 58.8 %| 83.8 %| 72.5 %| 53.8 %| 72.5 %| Ciprofloxacin (50 %)    |
| [33] | India     | Multicentre    | –       | 259      | E-test  | 44.7 %| 77.9 %| –     | –     | 32.8 %| –                       |
| [53] | India     | Kolkata        | 2000–2001| 67       | ADM     | 0.0 % | 85.1 %| –     | 7.5 % | 0.0 % | Furazolidone (0.0 %)    |
| [37] | India     | North India    | –       | 68       | ADM     | 11.8 %| 48.5 %| –     | 16.2 %| 17.6 %| Furazolidone (22.1 %)   |
| [34] | India     | Varanasi       | 2005–2006| 63       | ADM     | 4.7 % | 100.0 | 0.0 % | 65.1 %| –     | –                       |
| [32] | Pakistan  | Karachi        | 2005–2008| 178      | NM      | 36.0 %| 89.0 %| –     | 12.0 %| 37.0 %| Ofloxacin (185.8 %)     |
| [54] | Pakistan  | Karachi        | 2008–2013| 92       | E-test  | 5.4 % | 97.8 %| 16.2 %| 4.3 % | 2.2 % | Ofloxacin (301.2 %), Furazolidone (15.2 %) |
| [55] | Pakistan  | Karachi        | 2007–2009| 92       | E-test  | 32.6 %| 47.8 %| –     | –     | 2.2 % | –                       |
| [56] | Pakistan  | Karachi        | 2009–2010| 162      | E-test  | 37.0 %| –     | –     | –     | –     | –                       |
| [55] | Pakistan  | Rawalpindi     | 2011–2012| 46       | E-test  | 47.8 %| 73.9 %| –     | 4.4 % | 54.3 %| Ciprofloxacin (13.0 %)  |
| [57] | Bangladesh| Dhaka          | 1999–2001| 174      | ADM     | 10.0 %| 77.5 %| –     | 15.0 %| 6.6 % | –                       |
| [23] | Bangladesh| Dhaka          | 2014     | 56       | ADM     | 39.3 %| 94.6 %| 66.1 %| 0.0 % | 3.6 % | –                       |

Abbreviations: ADM Agar Dilution Method, DDM Disk diffusion method, E-test Epsilometer test, CAM clarithromycin, MNZ metronidazole, LVX levofloxacin, AMX amoxicillin, TCN tetracycline
values predominant (94.4 % of resistant strains showed 32 mg/L or more). Antibiotic resistance rate did not differ among different age groups, gender and clinical outcomes ($P >0.05$).

Overall, there was no strain resistant to all tested antibiotics. Only five strains were resistant to triple antibiotics; CAM, MNZ, and LVX (Table 3). Among all strains, 28.6 % (12/42) showed dual-drug resistance to MNZ and LVX. Additionally, three strains (7.1 %) were resistant to CAM and MNZ. No differences were observed in clinical outcomes between single-drug and multidrug resistant infections ($P >0.05$).

**Detection of *H. pylori* genes mutations associated with antimicrobial resistance**

The two and three MNZ-resistant strains did not show PCR identifiable specific bands target of *rdxA* and *frxA*, respectively. Therefore, a total 35 *rdxA* and 34 *frxA* of MNZ-resistant strains were analyzed in this study compared to 4-sensitive strains. Both of DNA sequence analysis of *rdxA* and *frxA* from MNZ-sensitive strains revealed intact reading frames (lacking nonsense mutations). Pairwise alignment identified that the MNZ-sensitive strains shared 94.5–97.3 % and 96.5–98.6 % identity with the reference strain, 26695 for *rdxA* and *frxA*, respectively. In contrast, most of the *rdxA* of MNZ-resistant strains contained missense mutations (12/37, 32.4 %) and nonsense mutation resulted premature stop codon (12/37, 32.4 %, Table 4). Moreover, *rdxA* alleles of 7 strains (18.9 %) contained nucleotide deletion and/or insertion that resulted in translational frameshift. The similar pattern with *rdxA* showed in *frxA* of MNZ-resistant that also contained missense mutations, premature stop codon and translational frameshift (11/37, 29.7 %; 4/37, 10.8 % and 17/37, 45.9 %, respectively). The association between these two genes was relative coincidence without a synergistic effect to increase MIC values.

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**Table 2** The distribution of antibiotic resistance of *H. pylori* Nepalese isolated strains by sex and age

| Antibiotic | All patients | Sex | Age (years) |
|------------|--------------|-----|-------------|
|            | (n = 42)     | Female (n = 26) | Male (n = 16) | <29 | 30–39 | 40–49 | 50–59 | >60 |
| AMX        | 0 (0.0)      | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| CAM        | 9 (21.4)     | 7 (26.9) | 2 (12.5) | 2 (20.0) | 2 (28.6) | 2 (18.2) | 1 (14.3) | 2 (28.6) |
| MNZ        | 37 (88.1)    | 24 (92.3) | 13 (81.3) | 8 (80.0) | 6 (85.7) | 9 (81.8) | 7 (100.0) | 7 (100.0) |
| TNC        | 0 (0.0)      | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| LVX        | 18 (42.9)    | 10 (38.5) | 8 (50.0) | 6 (60.0) | 2 (28.6) | 3 (27.3) | 2 (28.6) | 5 (71.4) |

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

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**Fig. 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.
Among 7 strains with high MIC values (>256 mg/L or more), 63.6 % strains were associated with frameshift mutation at position 18 of frxA (7/11) with or without rdxA involvement. Interestingly, there was no mutation on any rdxA and frxA in one strain with high MIC values (Nepal120).

Based on the previous report [10, 11], we performed NGS of the Nepal120 strain (average sequencing depth was 249.8× and overall %GC was 39.0). Nonetheless, we could not obtain ackA and rnc from NGS data. Using strain 26695 and the control MNZ-sensitive strain Nepal145, we could not identify any mutations in full-length frxA, dppB, rpsL, and rps4. In contrast, we revealed missense mutations in the full-length of rdxA at Arg-90, His-97, Pro-106 and Val-111. Moreover, we also confirmed involvement of novel mutated sequences in the form of amino acid substituted at Ala-212, Gln-382, Ile-485 of dppA and Leu-145, Thr-168, Glu-117, Val-121, Arg-221 in dapF.

There was no mutation on both of gyrA and gyrB subunits among the control four LVX-sensitive strains. Among 18 LVX-resistant strains, 17 had amino acid variants at gyrA subunit (Table 5). The major well-known point mutations in the 91- and 87-positions were predominant (15/18, 83.3 %), including 9 of LVX-resistant strains (50.0 %) substituted amino acid at Asp-91, while six strains had amino acid substitution at Asn-87 (33.3 %). Other mutations included substituted amino acid at Ala-88, Ser-63 and Arg-130. On the other hand, only one strain exhibited amino acid substitution at Glu-483 in gyrB subunits. However, it is coincidence with gyrB without influence to increase of MIC values. There was no correlation between degree of LVX-resistance with the type and number of mutations in both genes. Based on 23S rRNA sequenced in the 9 CAM-resistant strains exhibited 3 (44.4 %) and 2 (22.2 %) had point mutation specifically on A2143G and A2146G, respectively. In contrast, we identified minimal nucleotide variation on the CAM-sensitive strains. Interestingly, there was no 23S rRNA mutation in four strains with high MIC values (>256 mg/L or more). Based on the previous report [8], we also performed next generation sequencing of the Nepal90, Nepal110, Nepal114 and Nepal145 strains (average sequencing depth was 139.5×, 117.3×, 127.5×, 139.4×, respectively and overall %GC was 39.2, 39.0, 38.8, 38.9, respectively). Using strain 26695 and the control CAM-sensitive strain Nepal44, we could not identify any mutations in full-length 23S rRNA. We confirmed the involvement of novel mutated sequences in C113T and G20A of rpl22 and some interest mutations of infB such as G793A, C2669T, G2043T and C2784A (Table 6).

### Discussion

The AMX resistance rates in South Asia is diverse (Table 1), we revealed there was no AMX resistance from Nepalese isolates. Together with CAM or MNZ, AMX is the first-line regimen for treatment of *H. pylori* infection particularly as a secondary antibiotic in the low efficacy of CAM-based triple treatment zone [28–31]. Although in general the AMX resistance is rare, the increasing AMX primary resistance rates have been reported in the neighbor’s country; India and Pakistan [22, 32–35]. AMX is one of the most commonly used antibiotics in recent years in Nepal as similar as ceftriaxone and gentamycin [36]. Additionally we observed no resistance to TCN, in contrast to studies from India and Pakistan [22, 32, 35, 37]. TCN is used as a salvage quadruple therapy [28, 38] and may be a useful alternative first-line regimen in Nepal. A strict regulation for anti-microbial use is necessary to counteract failure of these two essential antibiotics in Nepal.

Importantly, we observed a high prevalence of CAM resistance (21.4 %) in Nepalese strains. It is overabundance of the breaking points required by the Maastricht guidelines on *H. pylori* infection management (>15–20 %) [38, 39], consequently, CAM-based regimen may insufficient as a first line treatment for *H. pylori* eradication in Nepal. A meta-analysis demonstrated that utilization of triple therapy that consist of PPI, AMX, and CAM in cases of CAM resistance diminished the treatment efficacy by 66 % [40]. CAM is not a drug of choice in Nepalese physicians related a high cost [41]. Nonetheless, other macrolides consumption such as erythromycin and azithromycin used for lower respiratory infection in Nepal [41] and become essential risk for cross-resistance to CAM [42]. Additionally, similar with other countries in Asia, there was emerging resistance to MNZ in Nepal. MNZ is a simple medication often utilized to treat different diseases, for example, intestinal parasites and periodontal and gynecologic [43, 44]. In Asia, only Japan, Thailand, and Malaysia have populations with <40 % MNZ resistance [5]. Therefore, regimens including MNZ are not suitable and should not be chosen as first-line treatment in Nepal.

The T2183C and A2223G transformations have been frequently found to be the reason of observed CAM
resistance in Asian countries than those in Europe and North America [45]. However, in Nepal we observed the contribution of interest point change on A2143G and A2146G, as previous reports [46, 47]. The A2143G mutation has a much stronger effect than the A2142G and A2142C mutations [46]. Interestingly among several strains with high MIC values (>256 mg/L or more) without 23S rRNA involvement, we confirmed novel mutated

| No | Strains | MIC (mg/L) | rdxA | frxA |
|----|---------|------------|------|------|
| 1  | 2       | 48         | 13frameshift | R86⁵ |
| 2  | 4       | >256       | Q11⁴ | 18frameshift |
| 3  | 5       | 64         | N73⁴ | R3T, 54frameshift |
| 4  | 8       | 64         | Q11⁴ | Q5⁴ |
| 5  | 14      | 32         | R16H, L62V, K190⁴ | P2E, R3P, M66l, A70V |
| 6  | 15      | 64         | K2N, 4frameshift | I44T, 47frameshift |
| 7  | 16      | 48         | E107R, 109frameshift | W137⁴ |
| 8  | 18      | 128        | C148Y | undetermined |
| 9  | 29      | >256       | R16H, A80T, S108A | 18frameshift |
| 10 | 34      | 64         | undetermined | 106frameshift |
| 11 | 41      | 16         | R16H, R41K, 43frameshift | G76R, A152V |
| 12 | 49      | >256       | C140Y | 18frameshift |
| 13 | 52      | >256       | G189S | undetermined |
| 14 | 55      | 24         | L62V, S108A, S196N, Q197⁴ | P41L, E176K |
| 15 | 61      | 12         | None | A15V, I144V, M66l |
| 16 | 64      | 32         | R16H, S108A, R176C, S196N | None |
| 17 | 70      | 32         | K60⁴ | D2e, A85V, K178N |
| 18 | 74      | 64         | S45G | 6frameshift |
| 19 | 83      | >256       | M21V, A80T, Q119⁷ | A70V |
| 20 | 84      | 16         | Q50⁴ | R58H |
| 21 | 86      | 96         | Q50⁴ | R25T, M66l, A154T |
| 22 | 89      | 32         | Q65⁴ | A115V |
| 23 | 90      | 64         | 45frameshift | 18frameshift |
| 24 | 92      | >256       | C140Y | 18frameshift |
| 25 | 94      | 64         | Q50⁴ | undetermined |
| 26 | 108     | 128        | R16L | 18frameshift |
| 27 | 110     | 32         | None | P41L |
| 28 | 113     | 128        | A40T | A16T, I44V, 70frameshift |
| 29 | 114     | >256       | Q16⁴ | 18frameshift |
| 30 | 116     | >256       | G163D | 18frameshift |
| 31 | 120⁶    | >256       | None | None |
| 32 | 123     | >256       | Q50⁴ | V6⁴ |
| 33 | 124     | >256       | D23G | 18frameshift |
| 34 | 137     | 128        | M56l, 201frameshift | 70frameshift |
| 35 | 140     | 96         | 60frameshift | 71frameshift |
| 36 | 141     | 64         | S43L | 72frameshift |
| 37 | 142     | 64         | undetermined | A15V |

Q11⁴ means premature stop codon at Gln11; 13frameshift means frameshift mutation in the amino acid 13; R16H means amino acid substituted at Arg-16; None means no specific mutation; Undetermined is the strains that failed to show identifiable specific bands of rdxA or frxA target in PCR

High MIC values strain without specific mutation in rdxA and frxA but contained mutation in dppA and dapF
sequences in rpl22 and infB in the different position than previous publication [8]. Suggesting that rpl22 and infB mutations might not only result in synergistic effects, but also could be independent causes of CAM resistance. On the other hand, we recognized diverse mutations involving the rdxA and frxA in the large part MNZ-resistant-strains; appear differently in relation to against MNZ-sensitive strains. Additionally, several strains with high MIC values were associated with a framing error in position 18 of frxA that may become a particular mutation site of Nepalese MNZ-resistant strains. Finally, we introduced the novel mutation in dppA and dapF in addition to rdxA mutations but irrespective of frxA and rpsL mutations. Unlike dapF which is associated with biosynthesis of lysine and peptidoglycan [48], dppA has a role in the transportation of dipeptide ATP-binding cassette on a drug efflux pump [11] that eventually lead to MNZ resistance.

Several guidelines proposed that LVX ought to be utilized as a part of rescue treatment based on antibiotic susceptibility testing [28, 38, 49]. However, our findings showed a high prevalence of primary resistance to LVX that may also prompt cross-resistance with other fluorquinolones. It is become a serious challenge and may reduce the efficacy of treatment with LVX-based regimens in Nepal. In addition, together with MNZ, LVX is the most commonly observed as multidrug resistance in Nepal. Furthermore, 5 strains were identified resistance to triple antibiotics. H. pylori strains harboring triple or quadruple resistance can hinder the choice and achievement of eradication regimens.

As similar with previous reports [50–52], point mutations at amino acid 87 (Asn to Lys, Tyr, or Ile) and 91 (Asp to Asn, Gly, or Tyr) were also mainly found for Nepalese strains. Interestingly, different transformations including substituted amino acid at Ser-63 and Arg-130 also associated with high MIC values. A few mutations and the coincidence of Glu-483 substitution in gyrA subunits with gyrB suggested a minimum influence of the gyrB mutations in Nepalese LVX-resistant strains. Finally, mutation analysis at position 18 of frxA, Asn-87 and/or Asp-91 of gyrA, A2143G and A2146G of 23SrRNA will be useful as guiding follow-up of eradication after first-line regimens failure in Nepal. Recently, it was created a high accuracy DNA strip genotyping test combining PCR and hybridization that allows the molecular identification of mutations in the gyrA and 23SrRNA within 6 h [47].

The number of samples in this study was relatively low, which certainly suggests the limitations of this study.

| Table 5 | MIC of levofloxacin resistant strains and the mutation of gyrA and gyrB genes |
|---------|---------------------------------------------------------------|
| No      | Strains | MIC (mg/L) | gyrA  | gyrB  |
| 1       | 2       | >32        | N87K  | None  |
| 2       | 5       | >32        | D91G  | None  |
| 3       | 8       | >32        | D91N  | None  |
| 4       | 16      | >32        | S63P, D91N | None |
| 5       | 18      | >32        | D91N  | None  |
| 6       | 29      | >32        | S63P, N87K, P188S | None |
| 7       | 38      | >32        | D99V  | None  |
| 8       | 49      | >32        | N87K, D91N, V172I | None |
| 9       | 55      | >32        | N87I  | E483K |
| 10      | 70      | >32        | None  | None  |
| 11      | 86      | >32        | N87K  | None  |
| 12      | 89      | >32        | D91N, R130K | None |
| 13      | 90      | >32        | N87K  | None  |
| 14      | 120     | 8          | A88P  | None  |
| 15      | 123     | >32        | D91Y  | None  |
| 16      | 140     | >32        | D91N  | None  |
| 17      | 141     | >32        | D91N  | None  |
| 18      | 142     | >32        | S63P, R130K | None |

N87K means amino acid substituted at Asn-87; None means no specific mutation

| Table 6 | MIC of clarithromycin resistant strains and the mutation of 23S rRNA gene |
|---------|---------------------------------------------------------------|
| No      | Strains | MIC (mg/L) | 23S rRNA | rpl22 | infB  |
| 1       | 5       | >256       | A2143G   |      |      |
| 2       | 29      | >256       | A2143G   |      |      |
| 3       | 49      | >256       | A2146G   |      |      |
| 4       | 89      | >256       | A2143G   |      |      |
| 5       | 90      | >256       | None     | C113T | C193A, T449C, G793A, T870G, C1157T, C1988T, C2669T, A2781G, C2784A |
| 6       | 92      | >256       | A2146G   |      |      |
| 7       | 110     | >256       | None     | None  | C133G, G139A, C821T, A2551G, 547del, 571del |
| 8       | 114     | >256       | None     | G20A  | A298G, G448A, G568A, A1108G, G2403T, C2669T |
| 9       | 145     | >256       | None     | G20A  | G8A, A403G, G793A, C810A, C878T, T1171G, G2043T, C2784A, G793A, C812T |

A2143G means point mutation at 2143 position; None means no specific mutation
In addition, we only determined the presence of well-known genetic mutations associated with antibiotic resistance. However, our results could as a susceptibility-guided treatment in Nepal. High prevalence of CAM, MNZ, and LVX resistance in Nepal results in prerequisite for utilizing other alternative strategies, for example, bismuth or non-bismuth-based quadruple regimens or rifabutin-based triple therapy is fundamental in Nepal (Table 7) [5]. Additional clinical trials are required to enhance the rate of successful eradication in Nepal.

**Conclusions**

We revealed the rates of resistance to CAM, MNZ, and LVX were high in Nepal, which recommends that CAM-, MNZ-, and LVX-based triple therapies are not useful as first-line treatment in Nepal. TCN can be still utilized, albeit local information regarding its successful eradication rate is inadequate. Bismuth or non-bismuth-based quadruple regimens, furazolidone-based triple therapy or rifabutin-based triple therapy may become alternative strategy after first-line regimens failure in Nepal.

**Additional file**

Additional file 1: Table S1. The oligonucleotide primers for amplifying \( rdxA, \) \( rfxA, \) \( gyxA, \) \( g_yxB \) and \( 23S \) \( rRNA. \) (DOCX 14 kb)

**Abbreviations**

AMX: Amoxicillin; CAM: Clarithromycin; MIC: Minimum inhibitory concentration; MNZ: Metronidazole; PCR: Polymerase chain reaction; PPI: Proton pump inhibitors; TCN: Tetracycline; TUTH: Tribhuvan University Teaching Hospital

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**Availability of data and materials**

The detail data and materials available on request (yyamaoka@oita-u.ac.jp). Nucleotide sequence data reported are available under the DDBJ accession numbers LC184279-LC184300, LC184302-LC184422 and LC184494-LC184511.

**Authors’ contributions**

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

**Competing interests**

Potential competing interests: The authors declare that they have no competing interests.
 Consent for publication
Written informed consent was obtained from all participants including the consent to publish.

Ethics approval and consent to participate
The study protocol was approved by the Ethics Committee of TUTH and Oita University Faculty of Medicine, Japan. Written informed consent was obtained from all participants including the consent to participate.

Author details
1Department of Environmental and Preventive Medicine, Oita University Faculty of Medicine, Japan. 2Department of Gastroenterology and Hepatology, Baylor College of Medicine and Michael DeBakey Veterans Affairs Medical Center, Houston, TX 77030, USA.
3Gastroentero-Hepatology Division, Department of Internal Medicine, Faculty of Medicine-Institute of Tropical Disease, Universitas Airlangga, Surabaya 60115, Indonesia.

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