

Alternative Eradication Regimens for *Helicobacter pylori* Infection in Indonesian Regions with High Metronidazole and Levofloxacin Resistance

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1 **Alternative Eradication Regimens for *Helicobacter pylori* Infection in Indonesian**
2 **Regions with High Metronidazole and Levofloxacin Resistance**

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4 Short title: Alternative *H. pylori* regimens in Indonesia

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13

14

1 **Abstract**

2 The prevalence of *Helicobacter pylori* resistance to metronidazole and clarithromycin is high
3 in Indonesia. Moreover, the increasing levofloxacin resistance rates in the absence of bismuth
4 treatment in Indonesia has led to the use of other antibiotics as alternative regimens. We
5 determined the minimum inhibitory concentrations (MICs) of five alternative antibiotics for
6 *H. pylori* (rifaximin, rifabutin, furazolidone, garenoxacin, and sitafloxacin) using the agar
7 dilution method and assessed mutations associated with antibiotic resistance using next-
8 generation sequencing. Analysis of 106 strains isolated from 1039 adult dyspeptic patients
9 revealed that none of the strains were furazolidone-resistant. All strains were also sensitive to
10 rifabutin and sitafloxacin. In contrast, the rates of resistance to rifaximin and garenoxacin
11 were high (38.9% and 6.7%, respectively). The strains isolated from patients on Java Island
12 had the highest resistance rates to garenoxacin and rifaximin. In addition, the resistance was
13 distributed evenly among the ethnic groups, ranging between 25.0% and 69.2%. Except for
14 rifaximin, for which the resistance rate was 38.9%, the other four antibiotics could be
15 successfully employed to eradicate levofloxacin- and metronidazole-resistant *H. pylori*
16 infections *in vitro*. Interestingly, garenoxacin-sensitive strains were found in regions with
17 high clarithromycin resistance rates such as Bali and Papua Islands. In contrast, rifaximin
18 might not be considered as an alternative antibiotic in regions with high clarithromycin
19 resistance. There was an inconsistent association between *gyrA* and *gyrB* mutations and
20 garenoxacin resistance. We confirmed that the I837V (replacement of isoleucine at position
21 837 with valine), A2414T/V, Q2079K and K2068R were the predominant *rpoB* point
22 mutations. There was an association between *vacA* genotypes of *H. pylori* and rifaximin
23 resistance ($P = 0.048$). In conclusion, furazolidone-, rifabutin-, and sitafloxacin-based
24 therapies might be considered as alternative regimens to eradicate *H. pylori* in Indonesia,
25 including regions with high metronidazole and clarithromycin resistance rates. Moreover,
26 sitafloxacin but not garenoxacin should be considered for eradication of levofloxacin-
27 resistant strains.

28 **Keywords:** Indonesia; drug resistance; *Helicobacter pylori*; antibiotics

1 **Introduction**

2 *Helicobacter pylori* eradication has led to a significant decrease in the incidence of gastric
3 cancer and can prevent its progression.^{1,2} The *H. pylori* eradication regimens established in the
4 Asia Pacific region and three countries in East Asia (Japan, South Korea and China) have been
5 summarized in the recent guidelines.³⁻⁶ Nevertheless, resistance to clarithromycin, which is
6 included in the first-line therapy for *H. pylori*, has recently emerged in several regions across
7 the globe.⁷⁻¹⁰ In addition, resistance to alternative regimens including metronidazole was
8 significantly associated with its frequent use.^{9,11} Moreover, high levofloxacin resistance was
9 reported in several countries in Asia.^{9,11,12} Based on the Maastricht Consensus, a suitable first-
10 line regimen is considered to be effective against *H. pylori* if the eradication rate is more than
11 90%,¹³ thus it can prevent secondary antibiotic resistance. However, further investigation is
12 warranted to assess the antibiotic sensitivity of *H. pylori* in patients with multiple treatment
13 failures in specific countries to determine the best rescue treatment regimens.

14 Indonesia, located in Southeast Asia, is the fourth most populous country in the world,
15 with a total population of approximately 260 million in 2017, which is composed of various
16 ethnic groups. Java, Sumatra, Papua, Kalimantan, and Sulawesi Island are the five main islands,
17 with half of the total population living on Java Island. Similar to other regions in Indonesia,
18 we previously reported high resistance to clarithromycin (21.4%) on Java Island, which is
19 more than the recommended limit of 15% by the Maastricht consensus.¹⁴ In addition, the
20 resistance rates to metronidazole and levofloxacin in Indonesian *H. pylori* strains are high
21 (46.8% and 31.2%, respectively). Importantly, the prevalence of *H. pylori* infection in
22 Indonesians, particularly among the major ethnic group of Javanese is low (2.4%)¹⁵,
23 highlighting the difficulties in isolating strains and conducting clinical trials on *H. pylori*
24 eradication in Indonesia. In addition, although dyspepsia is the fifth most common symptom
25 in an inpatient setting in Indonesia, the availability of gastrointestinal endoscopy is limited,
26 and it is predominantly utilized on Java Island.¹⁶ Moreover, the Indonesian strains were
27 previously reported to harbor specific virulence factors associated with clinical outcomes.¹⁷
28 Therefore, *in vitro* analysis of the Indonesian *H. pylori* strains is important for determining

1 their susceptibility to salvage therapy and informing the clinicians on the best rescue regimens
2 for patients with multiple treatment failures.

3 Among the several antibiotics proposed as alternative regimens for *H. pylori* is
4 furazolidone, a synthetic nitrofurane with broad-spectrum antimicrobial activity that blocks
5 bacterial metabolism by interfering with bacterial oxidoreductase activity.¹⁸⁻²² Furthermore,
6 in a study, the sensitivity of *H. pylori* to rifabutin and the utility of rifabutin as a rescue
7 regimen following treatment failure with other antibiotics were reported in more than 50% of
8 the subjects.²³ Rifabutin is an anti-tuberculosis agent which acts on DNA-directed RNA
9 polymerase and inhibits transcription in *H. pylori*.²⁴⁻²⁶ Rifaximin is a semi-synthetic derivate
10 of rifamycin with antimicrobial activities against a broad spectrum of organisms, including *H.*
11 *pylori*, is not absorbed in the gastrointestinal tract, and associated with mutations in *rpoB*.²⁷
12 Conversely, garenoxacin and sitafloxacin, two novel quinolones, were proposed to treat *H.*
13 *pylori*-resistant strains harboring *gyrA* mutation.²⁸ In this study, we examined the resistance
14 profile of *H. pylori* to several antibiotics used as alternative regimens in a geographical area
15 with a high prevalence of clarithromycin- and metronidazole-resistant *H. pylori* strains. Our
16 findings suggest several potential regimens that might overcome the hurdle of clarithromycin
17 and metronidazole resistance and the results will important not only for Indonesia, but also
18 regional region and worldwide. Furthermore, we identified several point mutations in *H.*
19 *pylori* that might confer rifaximin resistance.

1 **Materials and methods**

2 **Patients and *H. pylori***

3 This nationwide study included 1039 adult dyspeptic patients who underwent endoscopic
4 biopsy between August 2012 and February 2016 in 18 cities on eight Indonesian islands.
5 Among these 1039 patients, 752 patients were reported in a previous study.¹⁴ Gastric biopsy
6 specimens of the remaining 287 patients from Cimacan (n = 22) and Surabaya City (n = 22)
7 on Java Island; Padang (n = 33), Palembang (n = 38), and Dolok Sanggul City (n = 47) on
8 Sumatera Island; Gunungsitoli (n = 32) on Nias Island; Kolaka (n = 50) on Sulawesi Island;
9 and Merauke (n = 43) on Papua Island were obtained in the current study. There were 599
10 males (age range, 17–88 years; mean, 46.14 ± 13.63 years) and 439 females (age range, 14–
11 80 years; mean, 47.79 ± 14.4 years). Patients with bleeding due to esophageal varices, those
12 with a history of partial gastric resection, and those with a history of successfully eradicated
13 *H. pylori* infection were excluded. Peptic ulcer disease was diagnosed by endoscopic
14 examination, whereas the diagnosis of gastritis was based on histologic examination. The
15 review board or the ethics committee of the following institutions reviewed and approved the
16 study protocol: Dr. Cipto Mangunkusumo Teaching Hospital (Jakarta, Indonesia), Dr.
17 Soetomo Teaching Hospital (Surabaya, Indonesia), Dr. Wahidin Sudirohusodo Teaching
18 Hospital (Makassar, Indonesia), and Oita University Faculty of Medicine (Yufu, Japan). All
19 study participants agreed to follow the study protocol and provided written informed consent.
20 *H. pylori* was isolated from homogenized antral biopsy specimens by inoculating onto
21 selective agar plates and incubating the plates up to ten days in microaerophilic environment
22 (10% O₂, 5% CO₂, and 85% N₂) at 37°C. The colonies that grew were sub-cultured onto
23 antibiotic-free Mueller-Hinton II agar (Beckton Dickinson, NJ, USA) supplemented with
24 10% horse blood under the same microaerophilic conditions. *H. pylori* isolates were
25 confirmed based on colony morphology and Gram staining as well as oxidase, catalase, and
26 urease test results. The isolates were stored in Brucella broth (Difco, NJ, USA) supplemented
27 with 10% dimethyl sulfoxide and 10% horse serum at –80°C.

28

1 **Antibiotic susceptibility testing**

2 The two-fold agar dilution method was used to determine minimum inhibitory concentrations
3 (MICs) of furazolidone (Tokyo Chemical Company, Tokyo, Japan), rifaximin (Tokyo
4 Chemical Company), rifabutin (Sigma Aldrich, St. Louis, MO, US), garenoxacin (Sigma
5 Aldrich), and sitafloxacin (Haoyuan Chemexpress, Shanghai, China). Briefly, the isolates
6 were sub-cultured on Mueller-Hinton II agar supplemented with 10% horse blood. The
7 bacteria were diluted in Brucella broth and adjusted to be equivalent to a McFarland opacity
8 standard of 3.0. The prepared bacterial suspension was then inoculated on Mueller-Hinton II
9 agar supplemented with blood. The MICs were determined after a 72-h incubation. An *H.*
10 *pylori* strain from American Type Culture Collection (catalog # 43504) was used as the
11 quality control. Resistance breakpoints were determined based on an MIC of >4 mg/L for
12 furazolidone and rifaximin and >1 mg/L for rifabutin, garenoxacin, and sitafloxacin, as
13 described previously.²⁹⁻³² The final concentrations of furazolidone and rifaximin ranged from
14 0.25 to 32 µg/mL, while those for rifabutin, garenoxacin, and sitafloxacin ranged from 0.064
15 to 8 µg/mL.

16
17 **Detection of virulence factors and resistant strains**

18 *H. pylori* DNA was extracted using the commercially available DNeasy[®] kit (Qiagen, Hilden,
19 Germany) and stored at -20°C until further analysis. Data on the *gvrA* and *gvrB* mutations in
20 *H. pylori* were available for the 752 patients that were reported in our previous publication.¹⁴
21 In addition, mutation analyses were performed for *gvrA* and *gvrB* mutation status in the
22 remaining 287 specimens. Furthermore, next-generation sequencing (MiSeq next-generation
23 sequencer; Illumina, San Diego, CA) was used to analyze all specimens for full-length *rpoB*,
24 *oipA* status (“on” or “off”), and the presence of *vacA* (s1 or s2; m1 or m2; and i1, i2, or i3),
25 *iceA* (*iceA1* or *iceA2*), *jhp0562*, and β -(1,3)*galT* genotypes of the Indonesian strains. The
26 BLAST algorithm implemented in the CLC Genomics Workbench software (ver. 11; Qiagen,
27 Venlo, Netherlands) was used for the analysis. The sequences of hp0701, hp0501, hpl198,
28 and hp0638 of the strain 26695 (GenBank accession number AE000511.1) were used as

1 queries to obtain the *gyrA*, *gyrB*, *rpoB*, and *oipA* sequences, respectively from the Indonesian
2 next-generation sequencing data. The variants related to antibiotic resistance were predicted
3 by comparing all the *rpoB* sequences of resistant strains and five random sensitive strains
4 with the *rpoB* sequence of the strain 26695 for rifaximin resistance and *gyrA* and *gyrB* for
5 garenoxacin and sitafloxacin resistance. Briefly, after obtaining the *rpoB*, *gyrA*, and *gyrB*
6 sequences and confirming the absence of insertions or deletions leading to frameshift
7 mutations, the sequences were aligned at the codon level using the MAFFT software
8 (<http://mafft.cbrc.jp/alignment/server/>). Subsequently, each codon of the resistant and
9 sensitive strains was compared to the reference sequence using our original PERL script and
10 confirmed by visual inspection. Variants found in both the resistant and the sensitive strains
11 were considered as normal variants and were excluded from further analysis. Variants found
12 in the resistant strains but not in the sensitive ones were considered as variants related to
13 antibiotic resistance.

14

15 **Statistical analysis**

16 Discrete variables were analyzed by the chi square test, whereas interval/ratio variables were
17 analyzed using Student's *t* test or the Mann-Whitney *U* test. *P* values of < 0.05 were
18 considered statistically significant. All statistical analyses were performed using the SPSS
19 statistical software package version 18.0 (SPSS, Chicago, IL, US).

20

1 **RESULTS**

2 **Resistance of *H. pylori* to alternative antibiotics**

3 Twenty-nine *H. pylori* strains were isolated from 287 patients including 1, 1, 10, 1, 7, and 9
4 strains from Surabaya, Palembang, Dolok Sanggul, Gunungsitoli, Kolaka, and Merauke,
5 respectively. No *H. pylori* strains could be isolated from the specimens obtained from the
6 patients in Cimacan and Padang. We also reanalyzed the 77 strains that were assessed for
7 their sensitivity to clarithromycin, amoxicillin, metronidazole, tetracycline, and levofloxacin
8 in our previous study.¹⁴ However, one of these strains (Malang1) did not grow properly.
9 Therefore, a total of 105 strains were analyzed in the current study.

10 Overall, more than half of the strains (61/105, 58.1%) were sensitive to all five
11 antibiotics examined in this study. Forty strains were resistant to rifaximin (38.9%, Table 1).
12 In addition, the rate of garenoxacin resistance was 6.7% (7/105). In contrast, none of the
13 examined strains exhibited resistance to furazolidone, rifabutin, or sitafloxacin. Four strains
14 were resistant to two antibiotics. The rates of strains resistant to rifaximin and garenoxacin
15 were higher in males than in females (28/67 [42.4%] vs. 12/38 [31.5%] and 5/67 [7.6%] vs.
16 2/38 [5.2%], respectively), although these differences were not statistically significant ($P =$
17 0.28 and $P = 0.65$, respectively). The antibiotic-resistant strains were more frequent among
18 those older than 30 years of age, albeit in the absence of a significant association. Overall, 95,
19 1, and 9 antibiotic-resistant strains were isolated from patients with chronic gastritis, gastric
20 cancer, and peptic ulcer, respectively. The rate of garenoxacin resistance was higher in the
21 patients with chronic gastritis than in those with peptic ulcer (6/95 [6.3%] vs. 0/9 [0.0%], $P =$
22 0.001)

23

24 **Rates of antibiotic resistance according to location and ethnicity**

25 The rate of garenoxacin resistance was highest among the *H. pylori* strains obtained from
26 Java Island compared to those from the other regions (15.4% vs. 10.0%, 6.2%, and 4.7%
27 from the Sumatera, Papua, and Sulawesi Island, respectively; Table 2). The garenoxacin
28 resistance was not detected in any of the strains from Kalimantan, Timor, and Bali. In

1 contrast, more than half of the strains isolated from the specimens of patients from
2 Kalimantan, Sulawesi, and Bali Islands had rifaximin resistance (60.0%, 52.4% and 50.0%,
3 respectively). Finally, the rate of rifaximin resistance was at least 20% on all the study
4 locations.

5 The analysis of the rates of antibiotic resistance according to ethnicity revealed that
6 the garenoxacin resistance rate of 20% was higher in the strains isolated from the Chinese
7 Indonesian patients than in those isolated from the Batakese, Buginese, and Papuan patients
8 (9.6%, 7.7%, and 6.2%, respectively; Table 3); however, this difference was not statistically
9 significant ($P = 0.44$, $P = 0.33$ and $P = 0.31$, respectively). None of the strains isolated from
10 the Ambonese, Balinese, Dayak, Javanese, Minahasanese, and Timor patients exhibited
11 garenoxacin resistance, indicating that the bacterial strains in the patients belonging to these
12 ethnic groups were sensitive to furazolidone, rifabutin, garenoxacin, and sitafloxacin but not
13 rifaximin. Only one strain from a Javanese patient (only 1 strain was isolated) was sensitive
14 to all five antibiotics. Rifaximin resistance were distributed evenly among ethnic group
15 (Table 3), ranging between 25.0% and 69.2% ($P = 0.81$). Within the ethnic groups with the
16 highest prevalence of *H. pylori* in Indonesia,¹⁵ all the strains were resistant to garenoxacin
17 and rifaximin, with the higher resistance rate to rifaximin observed in the Buginese patients
18 compared with that in the Papuan and Batakese patients (9/13 [69.2%] vs. 6/16 [37.5%]; $P =$
19 0.01 vs. 8/31 [25.8%], $P = 0.001$, respectively).

20

21 **Comparison of Alternative and Standard Antibiotic Regimens**

22 We determined the resistance rates of the 76 *H. pylori* strains reported in our previous study¹⁴
23 to the five antibiotics and compared them with the resistance rates to the standard antibiotics
24 used for *H. pylori* infection (Table 4). Our findings above indicated that, except for rifaximin
25 with a resistance rate of 35.5% (27/76), there was a possibility that the remaining four
26 antibiotics might overcome the high rate of resistance to levofloxacin and metronidazole.
27 Interestingly, *H. pylori* in the regions with high clarithromycin resistance rates, such as Bali
28 and Papua Islands (1/6, 16.7% and 1/7, 14.3%, respectively), was still sensitive to

1 garenoxacin, although this finding could be due to the low number of strains with
2 clarithromycin resistance. In contrast, the isolate from Java Island with the highest
3 clarithromycin resistance rate, also exhibited a high rate of rifaximin resistance. Thus, we
4 suggest that rifaximin should not be considered as an alternative in areas with high
5 clarithromycin resistance. Garenoxacin may combat *H. pylori* in regions with high
6 amoxicillin resistance such as Papua Island (0.0% vs. 14.3% of resistance rate for
7 garenoxacin and amoxicillin, respectively) but not in regions with high tetracycline resistance
8 such as Java Island (both 15.4% of resistance rate) (Table 4).

9 To further analyze the associations among resistance rates of metronidazole,
10 levofloxacin, garenoxacin, and rifaximin, we created a two-by-two table (Figure 1). Only
11 seven strains (9.2%) exhibited resistance to both rifaximin and levofloxacin. Furthermore, the
12 percentage of the levofloxacin-resistant/rifaximin-sensitive strains was lower than that of the
13 levofloxacin-sensitive/rifaximin-resistant strains (17/76 [22.4%] vs. 20/76 [26.3%]). In
14 contrast, the percentage of the metronidazole-resistant/rifaximin-sensitive strains was higher
15 than that of the metronidazole-sensitive/rifaximin-resistant strains (23/76 [30.3%] vs. 16/76
16 [21.1%]). Conversely, the percentages of the metronidazole-resistant/garenoxacin-sensitive
17 and the levofloxacin-resistant/garenoxacin-sensitive strains were higher than those of the
18 metronidazole-sensitive/garenoxacin-resistant and levofloxacin-sensitive/garenoxacin-
19 resistant strains (19/76 [25.0%] and 30/76 [39.5%] vs. 0/76 [0.0%] and 1/76 [1.3%],
20 respectively).

21

22 **Mutations associated with garenoxacin resistance**

23 We analyzed the *gyrA* and *gyrB* mutations from the two strains with high MICs for
24 garenoxacin identified in the current study (Merauke20 and Kolaka72, Table 5) together with
25 those identified in our previous study.¹⁴ Four strains with the highest MICs for garenoxacin
26 (2 mg/L) were associated with a high MIC for levofloxacin (>32 mg/L). In addition, three of
27 these strains had an amino acid substitution at Asp91 or Asn87 in the GyrA, which were
28 predominantly associated with the highest MICs for levofloxacin.¹⁴ However, the 13

1 garenoxacin-sensitive strains with low MIC values (<0.063-0.5 mg/L) were also associated
2 with those mutations, suggesting an inconsistent effect of these mutations. Moreover, none of
3 the garenoxacin-resistant strains harbored a substitution at Arg484 or Ser479 of the *gyrB*.
4 Finally, none of the strains harbored *parC* or *parE*, the two important genes associated with
5 quinolone resistance in other bacteria.

6

7 **Mutations associated with rifaximin resistance**

8 We analyzed full-length *rpoB* from 40 rifaximin-resistant strains based on the next-
9 generation sequencing data, with an average sequencing coverage ranging from 82.43x to
10 560.85x and a Q₃₀ score percentage ranging from 80.59% to 96.31% (Supplementary Table
11 1). Five random rifaximin-sensitive strains were used for comparison. Pairwise alignment
12 identified that the garenoxacin-sensitive strains shared 95.7%–97.8% identity with the
13 reference strain 26695. Using the strain 26695 and the garenoxacin-sensitive control strains,
14 DNA sequence analysis of *rpoB* from all rifaximin-sensitive strains revealed intact reading
15 frames that lacked nonsense mutations. Among all 2890 codons of *rpoB*, 1010 codons had
16 non-synonymous substitutions, indicating a change of nucleotide without a change in the
17 amino acid (silent mutations). In contrast, majority of the rifaximin-resistant strains (39/40
18 [97.5%]) contained missense mutations. We confirmed that the predominant point mutations
19 of *rpoB* were the replacement of isoleucine at position 837 with valine amino acid (8/40
20 [20%]), alanine at position 2414 with valine or threonine (8/40 [20%]), glutamine at position
21 2079 with lysine (7/40 [17.5%]), and lysine at position 2068 with arginine (7/40 [17.5%]).

22

18

23 **Virulence factors and antibiotic resistance types**

24 In addition to the data on virulence factors that we reported previously,¹⁴ we analyzed
25 virulence factors in the 29 newly identified *H. pylori* strains including *cagA*, *vacA*, *iceA*,
26 *jhp0562/β-(1,3)galT*, and *oipA* (Supplemental Table 1). There was association between the
27 *vacA* genotype of *H. pylori* with rifaximin resistance ($P = 0.048$). The genotypes s2m1 and
28 s1m1 of *vacA* tended to be more frequent in the garenoxacin-resistant strains compared with

1 the *vacA* s1m2 and s2m2 genotypes (2/2 [100.0%], 32/74 [43.2%], 6/26 [23.1%], and 0/2
2 [0.0%]; $P = 0.051$). There were no significant associations between other virulence factors
3 and antibiotic resistance.

4

5 **Nucleotide sequencing**

6 The nucleotide sequences were deposited in the DDBJ under accession number LC420353-
7 LC420380 (*vacA*), LC420381-LC420408 (*oipA*), LC420409-LC420436
8 (*jhp0562* and *jhp0563*), LC420437-LC420462 (*iceA*), LC420463-LC420466 (*gyrA* and *gyrB*)
9 and LC420467-LC420511 (*rpoB*).

10

1 **Discussion**

2 The current study revealed that none of the *H. pylori* strains isolated from Indonesian patients
3 were resistant to furazolidone, suggesting that furazolidone might be considered as an
4 alternative *H. pylori* treatment regimen in Indonesia, especially in regions with high rates of
5 strains exhibiting dual resistance to clarithromycin and metronidazole.³³ Our results are in
6 agreement with those reported by a study from a neighboring country, Malaysia, which also
7 found that all the isolated strains were sensitive to furazolidone.³⁴ Furazolidone use has been
8 proposed in recent guidelines for *H. pylori* management in developing countries, due to its
9 efficacy, low rate of primary bacterial resistance, and lack of alternative and low-cost
10 therapies.^{35,36} To improve the *H. pylori* cure rates, bismuth should be added to therapy. For
11 example, the addition of bismuth to quadruple therapy including furazolidone has been
12 successful in China, with cure rates reaching 92.26% with minimal side effects.³⁷ However,
13 the unavailability of bismuth in certain regions³⁸ due to potential bismuth-associated
14 carcinogenic effects, including mutagenicity and genotoxicity in *in vitro* and animal models,
15 ^{39,40} it has been classified as a type III carcinogen for humans in 1997 by the International
16 Agency on Research on Cancer. Furthermore, there are currently no standardized rescue
17 therapies available for patients who fail the initial furazolidone-based treatment.

18 Our finding of all isolated strains exhibiting rifabutin sensitivity provides support for
19 rifabutin as a potential alternative antibiotic against *H. pylori*. The concentrations of rifabutin
20 in the gastric juice were reported to be 10–17 times higher than in peripheral blood.⁴¹ The
21 antibacterial activity of rifabutin, which is not affected by the low pH environment in the
22 stomach, is higher than that of rifampicin.²⁴ Importantly, its target is different from that of
23 clarithromycin. Therefore, its efficacy in strains with primary clarithromycin resistance, even
24 in those that are also resistant to metronidazole, is high^{42,43}, although the majority of the
25 clinical trials to define these differences were conducted in Western countries. Nonetheless,
26 the adverse effects of rifabutin such as myelotoxicity should be considered. Furthermore, the
27 increased use of rifabutin in Indonesia, a country with high tuberculosis prevalence, might
28 lead to rifabutin resistance of *Mycobacterium tuberculosis*. Several studies reported

1 substantial *in vitro* cross-resistance to rifampicin, a main component of the tuberculosis
2 therapy regimens, although rifabutin resistance in *H. pylori in vitro* was rarely reported.²⁵ A
3 history of rifampicin treatment should be taken into consideration before prescribing rifabutin
4 for *H. pylori* eradication to reduce the possibility of treatment failure for tuberculosis and *H.*
5 *pylori* eradication. Importantly, rifabutin use in combination with clarithromycin should be
6 avoided, based on evidence showing the inhibition of rifabutin metabolism by clarithromycin
7 in liver microsomes⁴⁴, which suggests that potential toxicity might arise with combination
8 use.

9 Compared with the other fluoroquinolones, sitafloxacin is a more potent inhibitor of
10 DNA gyrase and topoisomerase IV, which play important roles in bacterial DNA repair,
11 transcription, replication, and recombination.⁴⁵ Sitafloxacin improves the efficacy of
12 quinolone-based rescue therapy by virtue of its ability to eradicate *H. pylori* strains with *gyrA*
13 mutations.⁴⁶ However, the limited access and availability are the main concerns regarding
14 sitafloxacin. Currently, Japan and Thailand are the only countries that provide sitafloxacin in
15 their healthcare system, and clinical trials for sitafloxacin are underway in Western
16 countries.⁴⁷ In contrast, although garenoxacin was also reported to eradicate *H. pylori* strains
17 with *gyrA* mutations⁴⁸, there were several strains that were resistant to this antibiotic in the
18 current study. Interestingly, the MIC value of levofloxacin was not associated with MIC value
19 for garenoxacin. For example, although all seven garenoxacin-resistant strains exhibited the
20 highest MICs for levofloxacin (>32 mg/L), all ten strains with the highest levofloxacin MIC
21 were sensitive to garenoxacin. The lower antibacterial activity of garenoxacin against *H.*
22 *pylori* compared with that of sitafloxacin might be associated with the high affinity of
23 sitafloxacin to DNA gyrase.⁴⁹ Due to high levofloxacin resistance rates in Indonesia, our
24 finding should be instrumental in formulating second-line regimen guidelines to eradicate *H.*
25 *pylori*.

26 One study found that double mutations in *gyrA* were associated with a seven-fold
27 increase in sitafloxacin MIC compared with the pretreatment MICs and that double mutations
28 in *gyrA*, including the mutations at Asp91 and Asn87, were associated with eradication

1 failure.⁵⁰ We found that double mutations were not associated with an increase in the
2 sitafloxacin MIC, although none of the strains harbored both Asp91 and Asn87 mutations.
3 Similar to sitafloxacin, none of the single or double mutations in GyrA or GyrB were
4 associated with garenoxacin resistance. Although *parC* and *parE* are important genes
5 associated with quinolone resistance, none of the isolated *H. pylori* strain exhibited the
6 presence of/expressed these genes, as previously described.⁵¹ Our results suggest that gene/s
7 other than *gyrA* or *gyrB* were associated with resistance to sitafloxacin and garenoxacin,
8 which should be investigated in future studies.

9 Among the several alternative drugs tested in the current study, the rate of resistance
10 was highest to rifaximin; this finding is in agreement with a previous study showing that
11 rifaximin-based triple therapy did not achieve acceptable *H. pylori* cure rates.^{52,53} However,
12 rifaximin is a promising *H. pylori* drug due to poor absorbance in the blood, which can
13 minimize adverse effects, and its higher bioavailability in the gastrointestinal tract that that
14 of other antibiotics.⁵⁴ The poor eradication rates might be due to a failure in achieving
15 sufficient therapeutic concentrations under and within the gastric mucosal layer, which is a
16 frequent site of *H. pylori* colonization.⁵⁵ Therefore, well-designed clinical trials are necessary
17 to evaluate rifaximin efficacy against *H. pylori*, including high-dose regimens of longer
18 duration, additional bioadhesive formulations, and combinations with mucolytic agents for
19 persistent coverage of the gastric mucosa.⁵⁵

20 Although *rpoB* mutations were reported to play a role in rifaximin resistance in other
21 bacteria such as *Escherichia coli*⁵⁶, *Clostridium difficile*^{57,58}, *Staphylococci*,⁵⁹ and *M.*
22 *tuberculosis*⁶⁰, only one study found an association between codons 524–545 and 585 of
23 *rpoB* with rifabutin resistance in *H. pylori*.²⁷ In the current study, we found numerous
24 missense mutations in *rpoB* of rifaximin-resistant strains, including novel and predominant
25 mutations: 837I, 2414A, 2079K, and 2068K. Although the mechanism remains unclear, the
26 risk for horizontal transmission of the *rpoB* mutations is lower than that of a resistance gene
27 located on a plasmid or transposon; however, certain yet-to-be determined conditions and
28 improper prescription or usage of antibiotic could still facilitate the rapid transmission of

1 such mutations.⁵⁹ Strict control should be practiced to prevent rifaximin failure in *H. pylori*
2 eradication.

3 The major limitation of this study was the relatively small number of samples.
4 However, these samples, obtained from 1039 endoscopic patients, comprised the biggest
5 cohort of *H. pylori* strains isolated in Indonesia thus far. In addition, only a fraction of the
6 genomic changes that were related to drug resistance, among a total of 1,600 genes of *H.*
7 *pylori*, were examined in the current study. Although sitafloxacin is a potent drug for *H.*
8 *pylori*, it has not been approved by Indonesia National Agency of Drug and Food Control.
9 Thus, sitafloxacin-based regimens cannot be currently prescribed in Indonesia.

10

11 **Conclusions**

12 Furazolidone-, rifabutin-, and sitafloxacin-based therapies should be considered as alternative
13 regimens to eradicate *H. pylori* in Indonesia, including regions with high rates of
14 metronidazole and clarithromycin resistance. Moreover, sitafloxacin but not garenoxacin
15 could inhibit the levofloxacin-resistant *H. pylori* strains.

16

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3

4 **Authors' contributions**

5 Conceived and designed the experiments: YY, MM, and AFS. Performed the experiments:

6 PS, MM, TU, LAW, and DD. Analyzed the data: MM, YY, LAW, RS, JA, DD, and KAF.

7 Contributed reagents/materials/analysis tools: AFS, IAN, MIL, DM, LHZ, FA, WBU, DS,

8 IDNW, JBW, AMJS, FY, SM, PA, UM, HM, YAAR, PS, N, and DR. Wrote the paper: MM,

9 YY, and AFS.

10

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23 **Potential competing interests**

24 The authors declare that they have no competing interests.

25

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- 19

1 Table 1. Rates of resistance to alternative antibiotics in *H. pylori* strains isolated in
 2 Indonesia

Characteristic	N	Resistance (%)				
		Furazolidone	Sitafloxacin	Garenoxacin	Rifaximin	Rifabutin
Total	105	0 (0.0)	0 (0.0)	7 (6.7)	40 (38.9)	0 (0.0)
Sex						
Male	67	0 (0.0)	0 (0.0)	5 (7.6)	28 (42.4)	0 (0.0)
Female	38	0 (0.0)	0 (0.0)	2 (5.2)	12 (31.5)	0 (0.0)
Age (years)						
17–30	12	0 (0.0)	0 (0.0)	0 (0.0)	3 (25.0)	0 (0.0)
31–40	13	0 (0.0)	0 (0.0)	1 (7.6)	5 (38.4)	0 (0.0)
41–50	28	0 (0.0)	0 (0.0)	2 (7.1)	12 (42.8)	0 (0.0)
51–60	34	0 (0.0)	0 (0.0)	3 (8.8)	13 (38.2)	0 (0.0)
>60	18	0 (0.0)	0 (0.0)	1 (5.5)	6 (33.3)	0 (0.0)
Clinical Outcome						
Gastritis	95	0 (0.0)	0 (0.0)	6 (6.3)	34 (35.7)	0 (0.0)
PUD	9	0 (0.0)	0 (0.0)	0 (0.0)	3 (33.3)	0 (0.0)
Cancer	1	0 (0.0)	0 (0.0)	1 (100)	1 (100)	0 (0.0)

3 Abbreviations: PUD, peptic ulcer disease

4

1 Table 2. Rates of resistance to alternative antibiotics in *H. pylori* strains isolated in
 2 specific regions of Indonesia

Region	N	Resistance (%)				
		Furazolidone	Sitafloxacin	Garenoxacin	Rifaximin	Rifabutin
Bali	6	0 (0.0)	0 (0.0)	0 (0.0)	3 (50.0)	0 (0.0)
Java	13	0 (0.0)	0 (0.0)	2 (15.4)	4 (30.7)	0 (0.0)
Kalimantan	5	0 (0.0)	0 (0.0)	0 (0.0)	3 (60.0)	0 (0.0)
Papua	16	0 (0.0)	0 (0.0)	1 (6.2)	6 (37.5)	0 (0.0)
Sulawesi	21	0 (0.0)	0 (0.0)	1 (4.7)	11 (52.4)	0 (0.0)
Sumatera*	30	0 (0.0)	0 (0.0)	3 (10.0)	7 (23.3)	0 (0.0)
Timor	14	0 (0.0)	0 (0.0)	0 (0.0)	6 (42.8)	0 (0.0)

3 *Strains obtained from patients from Nias Island was combined with those of Sumatera Island
 4 due to the low sample number.

5

6

1 Table 3. Prevalence of antibiotic resistance in *H. pylori* isolates based on ethnicity

Ethnicity	Island	N	Resistance (%)				
			FUR	SIT	GAR	RFX	RFB
Ambonese	Java	4	0 (0.0)	0 (0.0)	0 (0.0)	2 (50.0)	0 (0.0)
Batakese	Sumatera and Java	31	0 (0.0)	0 (0.0)	3 (9.6)	8 (25.8)	0 (0.0)
Balinese	Bali	6	0 (0.0)	0 (0.0)	0 (0.0)	3 (50.0)	0 (0.0)
Buginese	Sulawesi	13	0 (0.0)	0 (0.0)	1 (7.7)	9 (69.2)	0 (0.0)
Chinese	Java and Kalimantan	10	0 (0.0)	0 (0.0)	2 (20.0)	3 (30.0)	0 (0.0)
Dayak	Kalimantan	2	0 (0.0)	0 (0.0)	0 (0.0)	1 (50.0)	0 (0.0)
Javanese	Java	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Minahasanese	Sulawesi	8	0 (0.0)	0 (0.0)	0 (0.0)	2 (25.0)	0 (0.0)
Papuan	Papua	16	0 (0.0)	0 (0.0)	1 (6.2)	6 (37.5)	0 (0.0)
Timor	Timor	14	0 (0.0)	0 (0.0)	0 (0.0)	6 (42.8)	0 (0.0)

2 Abbreviations: FUR, furazolidone; STX, sitafloxacin; GAR, garenoxacin;

3 RFX, rifaximin; RIF, rifabutin.

1 Table 4. Comparison of the five alternative antibiotics with the standard regimens as reported by Miftahussurur et al ¹⁴

Island	n	Resistant Regiment (%)									
		CAM	AMX	MNZ	TCN	LVX	FUR	STX	GAR	RFX	RIF
Total	76	7 (9.1)	4 (5.2)	36 (46.7)	2 (2.6)	24 (31.2)	0 (0.0)	0 (0.0)	5 (6.5)	27 (35.5)	0 (0.0)
Bali	6	1 (16.7)	0 (0.0)	2 (33.3)	0 (0.0)	1 (16.6)	0 (0.0)	0 (0.0)	0 (0.0)	3 (50.0)	0 (0.0)
Java	13	3 (23.0)	0 (0.0)	7 (46.1)	2 (15.4)	7 (53.8)	0 (0.0)	0 (0.0)	2 (15.4)	4 (30.7)	0 (0.0)
Kalimantan	5	0 (0.0)	0 (0.0)	1 (20.0)	0 (0.0)	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (60.0)	0 (0.0)
Papua	7	1 (14.3)	1 (14.3)	3 (42.9)	0 (0.0)	2 (28.6)	0 (0.0)	0 (0.0)	0 (0.0)	2 (28.5)	0 (0.0)
Sulawesi	13	1 (7.7)	1 (7.7)	4 (30.8)	0 (0.0)	2 (15.4)	0 (0.0)	0 (0.0)	0 (0.0)	6 (46.1)	0 (0.0)
Sumatera	18	1 (5.6)	1 (5.6)	16 (88.9)	0 (0.0)	8 (44.4)	0 (0.0)	0 (0.0)	3 (16.7)	3 (16.7)	0 (0.0)
Timor	14	0 (0.0)	1 (7.1)	3 (21.4)	0 (0.0)	3 (21.4)	0 (0.0)	0 (0.0)	0 (0.0)	6 (42.8)	0 (0.0)

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

3 STX, sitafloxacin; GAR, garenoxacin; RFX, rifaximin; RIF, rifabutin.

1 Table 5. Mutations associated with quinolones

No	Strains	<i>gyrA</i> mutation	<i>gyrB</i> mutation	MIC	MIC	MIC
				LVX (mg/L)	GAR (mg/L)	SIT (mg/L)
1	Jayapura1	N87K	None	>32	0.25	0.063
2	Jayapura21	N87K	None	>32	0.125	0.063
3	Kupang2	D91N, A129T	S479G	4	0.125	<0.063
4	Kupang11	D91Y	None	>32	0.25	<0.063
5	Kupang23	A129T	S479G	>32	0.125	<0.063
6	Kupang41	D91N	R484K	8	0.5	<0.063
7	Malang1 ^s	D91N	None	16	n.a	n.a.
8	Manado18	None	None	8	0.5	<0.063
9	Manado20	D91Y	None	8	0.25	<0.063
10	Medan3	N87I	None	>32	2	0.25
11	Medan 10	None	None	25	<0.063	<0.063
12	Medan15	R140K, D192N	None	>32	2	0.5
13	Medan 17	D34N	None	16	0.5	<0.063
14	Medan18	D91G, D161N	None	4	<0.063	0.063
15	Medan22	D91N	None	>32	0.5	0.125
16	Medan 23	D34Y, R140K	None	4	0.063	<0.063
17	Medan30	D91N	None	>32	1	0.125
18	Pontianak50	D91G	None	>32	0.063	<0.063
19	Surabaya71	D91N	None	>32	2	0.25
20	Surabaya79	N87Y	R484K	>32	0.5	<0.063
21	Surabaya137	N87K	None	>32	0.5	<0.063
22	Surabaya151	N87K	None	>32	0.5	0.125
23	Surabaya283	D91Y	None	>32	2	<0.063
24	Surabaya304	D91G	None	>32	0.5	0.063
25	Merauke20 [‡]	E103G	None	n.a.	1	<0.063
26	Kolaka72 [‡]	D91G	None	n.a.	1	<0.063

- 1 ‡ The new strains with high garenoxacin minimum inhibitory concentrations (MICs) that
- 2 were not reported in our previous study
- 3 § One of the previously isolated strains could not sustain growth.
- 4 An MIC >1 mg/L was used as a resistance breakpoint for levofloxacin, garenoxacin, and
- 5 sitafloxacin.

1 Table 6. Mutations associated with rifaximin resistance

No	Strain Name	MIC (mg/L)	Mutation
1	Surabaya47	4	S355Y, I741V, T2002M, Q2079K
2	Jayapura21	4	V1125I, A2454V
3	Jayapura06	8	L547F, K786R, I837V, A964T, V1275I, A1533S, P1623S, D1697N, G1908E, A2099T, S2640Y
4	Jakarta9	4	I64V, L295I, S355Y, V657I, T1023I, S1197A, Q2042R, K2068R
5	Kupang10	4	G523C, I832V, E877K, K1006E, E1528D, Q1666H, N1944S, A2255V, G2512S, S2619I, V2774M
6	Kupang23	4	L169S, S355H, A693T, I837V, K854R, L977I, I1351T, N1999H, K2068R, Q2079K, S2415N, I2481V, V2528L, P2679S, M2696T
7	Kupang26	4	L169A, S355H, I837V, L977I, N1999H, Q2079K, S2415N, A2472V, I2481V, V2528L, P2679S, M2696T
8	Kupang29	4	K42R, I748V, T773I, A958T, E969D, S986G, A1025V, V1052I, V1122I, A2414V, S2619I
9	Kupang30	4	S355H, S627N, I837V, A1025V, D1162N, K1165R, L1401I, R1711H, Q2079K, A2234T, D2380E
10	Kupang34	4	S355H, A732V, I837V, V955I, L977I, V1028A, N1999H, K2068R, Q2079K, S2415N, I2481V, V2528L, P2679S, M2696T
11	Bangli42	4	I141V, E163D, N642D, R954W, E996G, M1264I, E1407K, Y2275C, K2462E, V2469I, G2480R, T2539A,
12	Bangli47	4	L314F, E1151K, I1190T, R1711H, K2068R, Y2326C, K2418Q, V2528L, T2536A, F2537L, K2538S, K2557R, V2561M, A2570T, S2734G
13	Bangli64	4	R63H, E162K, L295I, I336T, M667I, A735I, P816S, I837V, V867A, R973H, T975I, Q1010R, E1572Q, A1691V, A2346T, S2390G, G2491D, A2541V
14	Manado29	4	D255N, P259S, A1168V, A1181V, A1533T, L1765F, V1939I, A1950T,

			L2328I, R2694H, I2824V
15	Manado31	8	K307E, R984H, V1028A, A1533T, A2255V, Y2326C, A2494T
16	Merauke20	4	K786R, A964T, R2313H, G2512S
17	Merauke21	4	A1181V, G2180S, A2472T, P2545S, V2664M
18	Merauke27	4	I140V, A1533T, S1701N, A2505T, V2664M
19	Merauke37	4	S78P, P95H, M313L, K786R, A964T, L977F, E1014K, M1242I, D1379N, A1533S, G2180S, A2414T, R2477H, A2494T, I2730V
20	Kolaka72	4	R274I, S355Y, V538I, T635A, R1248H, G2403S, A2541, N2602D, R2641K, D2788G
21	Kolaka79	4	-
22	Kolaka96	4	R708K, L982S, E1161K, N1709D, E2382K, V2469I, R2477Y, V2528L, T2536A, F2537L, K2538S, K2557R, V2561M, R2882K
23	Kolaka98	4	P259S, A735T, S743A, I837V, T975S, A1025S, K1165R, D1379Y, K2068R, Q2079K, K2421R
24	Kolaka99	4	L295I, S355Y, A473V, M1175I, R1711H, Q2079K, A2454V, P2612S, S2675G
25	Makasar31	8	L295I, I336T, G615D, A735V, I837V, Q1010R, D1379Y, V1491I, H1985Y, V2237M, A2317V, D2380E, R2506C
26	Makasar45	4	E1161K, A2414V
27	Makasar52	4	E1161K, A2414V
28	Makasar55	4	P931S, A1643M, A1950T, K2068R, D2380E
29	Medan56	4	V303I, K1540N, A2414V, A2454V
30	Medan67	4	A497T, A958T, N1598H, T2002M, R2313C, A2414V
31	Medan75	4	I512V, M667I, A964V, T1402M, A2414V, E2599A, V2638I, S2791G
32	Padang42	4	H153Y, P931S, H1985Y, E2183K, A2414V, E2604G
33	Pontianak44	4	A487T, E969D, V1291I, A2255V, V2447I
34	Pontianak50	4	S355Y, S627N, E969K, R1563K, M1627I, A1676V, S1794N, N1999H, V2037I, D2449N, A2459T, T2533M, M2696T, E2859G

35	Pontianak5	4	V2802L
36	Surabaya283	4	P259S, T440A, A497T, E1232D, N1944S, V2037I, I2428V, I2564V, Y2740H, K2889R
37	Surabaya304	8	S78A, S355Y, K398R, V657I, E1486D, D2226S, L2328I, A2357V,
38	Medan15	4	A473V, Q991R, E1059G, S1197A, K2068R
39	Medan22	32	I66V, L295I, S355Y, I586L, E655K, V657I, G1620S, A2541T, L288II
40	Medan25	8	V52I, I66V, E106G, V657I, A756V, D2380E, K2482R

1 S355Y means Tyrosine replaced Serine amino acid in the position 355.

2 MIC, minimal inhibitory concentration

3

1 **Figure legends**

- 2 Figure 1. The associations among rates of resistance to metronidazole, levofloxacin,
3 garenoxacin, and rifaximin

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