Infection and Drug Resistance

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ORIGINAL RESEARCH

Alternative eradication regimens for *Helicobacter pylori* infection in Indonesian regions with high metronidazole and levofloxacin resistance

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Background: The prevalence of *Helicobacter pylori* resistance to metronidazole and clarithromycin is high in Indonesia. Moreover, the increasing levofloxacin resistance rates in the absence of bismuth treatment in Indonesia has led to the use of other antibiotics as alternative regimens.

Methods: We determined the minimum inhibitory concentrations (MICs) of five alternative antibiotics for *H. pylori* (rifaximin, rifabutin, furazolidone, garenoxacin, and sitafloxacin) using the agar dilution method and assessed mutations associated with antibiotic resistance using next-generation sequencing.

Result: Analysis of 106 strains isolated from 1039 adult dyspeptic patients revealed that none of the strains were furazolidone-resistant. All strains were also sensitive to rifabutin and sitafloxacin. In contrast, the rates of resistance to rifaximin and garenoxacin were high (38.9% and 6.7%, respectively). The strains isolated from patients on Java Island had the highest resistance rates to garenoxacin and rifaximin. In addition, the resistance was distributed evenly among the ethnic groups, ranging between 25.0% and 69.2%. Except for rifaximin, for which the resistance rate was 38.9%, the other four antibiotics could be successfully employed to eradicate levofloxacin- and metronidazole-resistant *H. pylori* infections in *vitro*. Interestingly, garenoxacin-sensitive strains were found in regions with high clarithromycin resistance rates such as Bali and Papua Islands. In contrast, rifaximin might not be considered as an alternative antibiotic in regions with high clarithromycin resistance. There was an inconsistent association between *gyrA* and *gyrB* mutations and garenoxacin resistance. We confirmed that the I837V (replacement of isoleucine at position 837 with valine), A2414T/V, Q2079K and K2068R were the predominant *rpoB* point mutations. There was an association between *vacA* genotypes of *H. pylori* and rifaximin resistance ($P = 0.048$).

Conclusion: furazolidone-, rifabutin-, and sitafloxacin-based therapies might be considered as alternative regimens to eradicate *H. pylori* in Indonesia, including regions with high metronidazole and clarithromycin resistance rates. Moreover, sitafloxacin but not garenoxacin should be considered for eradication of levofloxacin-resistant strains.

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

Introduction

Helicobacter pylori eradication has led to a significant decrease in the incidence of gastric cancer and can prevent its progression.[1,2] The *H. pylori* eradication regimens established in the Asia-Pacific region and three countries in East Asia (Japan, South Korea, and China) have been summarized in the recent guidelines.[3–6] Nevertheless, resistance to clarithromycin, which is included in the first-line therapy for *H. pylori*, has recently emerged in several regions across the globe.[7–10] In addition, resistance to alternative regimens including metronidazole was significantly associated with their

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frequent use.[9,11] Moreover, high levofloxacin resistance was reported in several countries in Asia, and this even reached a rate of up to 67%.[9,11–15] Based on the Maastricht Consensus V, a suitable first-line regimen is considered to be effective against *H. pylori* if the cure rate is >90%,[16] and thus, it can prevent secondary antibiotic resistance. However, further investigation is warranted to assess the antibiotic sensitivity of *H. pylori* to overcome the multiple treatment failures, with *H. pylori* eradication failure in >20% of cases, in specific countries to determine the best rescue treatment regimens.[17]

Indonesia, located in Southeast Asia, is the fourth most populous country in the world, with a total population of ~260 million in 2017, which is composed of various ethnic groups. Java, Sumatra, Papua, Kalimantan, and Sulawesi Island are the five main islands, with half of the total population living on Java Island. Similar to other regions in Indonesia, we previously reported high resistance to clarithromycin (21.4%) on Java Island, the rate of which is more than the limit of 15% recommended by the Maastricht consensus.[18] In addition, the resistance rates to metronidazole and levofloxacin in Indonesian *H. pylori* strains are high (46.8% and 31.2%, respectively). Importantly, the prevalence of *H. pylori* infection in Indonesians, particularly among the major ethnic group of Javanese, is low (2.4%),[19] highlighting the difficulties in isolating strains and conducting clinical trials on *H. pylori* eradication in Indonesia. In addition, although dyspepsia is the fifth most common symptom in an inpatient setting in Indonesia, the availability of gastrointestinal endoscopy is limited, and it is predominantly utilized on Java Island.[20]

Among the several antibiotics proposed as alternative regimens for *H. pylori* is furazolidone, a synthetic nitrofuran with broad-spectrum antimicrobial activity that blocks bacterial metabolism by interfering with bacterial oxidoreductase activity.[21–25] Furthermore, in a study, the sensitivity of *H. pylori* to rifabutin and the utility of rifabutin as a rescue regimen following treatment failure with other antibiotics were reported in >50% of the subjects.[26] Rifabutin is an antituberculosis agent which acts on DNA-directed RNA polymerase and inhibits transcription in *H. pylori*.[27–29] Rifaximin is a semisynthetic derivate of rifamycin with antimicrobial activities against a broad spectrum of organisms, including *H. pylori*, is not absorbed in the gastrointestinal tract, and associated with mutations in *rpoB*.[30] Conversely, garenoxacin and sitafloxacin, two novel quinolones, were proposed to treat *H. pylori*-resistant strains harboring *gyrA* mutation.[31] In this study, we examined the resistance profile of *H. pylori* to several antibiotics used as alternative regimens in a geographical area with a high prevalence of clarithromycin- and metronidazole-resistant *H. pylori* strains. Our findings suggest several potential regimens that might overcome the hurdle of clarithromycin and metronidazole resistance, and the results might be of value not only for Indonesia but also for countries worldwide. Furthermore, we identified several point mutations in *H. pylori* that might confer rifaximin resistance.

Materials and methods

Patients and *H. pylori*

This nationwide study included 1,039 adult dyspeptic patients who underwent endoscopic biopsy between August 2012 and February 2016 in 18 cities on eight Indonesian islands. Among these 1,039 patients, 752 were reported in a previous study.[18]Gastric biopsy specimens of the remaining 287 patients used in the current study (Figure 1) were obtained from the following regions: Cimacan (n=22) and Surabaya (n=22) on Java Island; Padang (n=33), Palembang (n=38), and Dolok Sanggul (n=47) on Sumatera Island; Gunungsitoli (n=32) on Nias Island; Kolaka (n=50) on Sulawesi Island; and Merauke (n=43) on Papua Island. There were 599 males (age range, 17–88 years; mean, 46.14±13.63 years) and 439 females (age range, 14–80 years; mean, 47.79±14.4 years). Patients with bleeding due to esophageal varices, those with a history of partial gastric resection, and those with a history of successfully eradicated *H. pylori* infection were excluded.

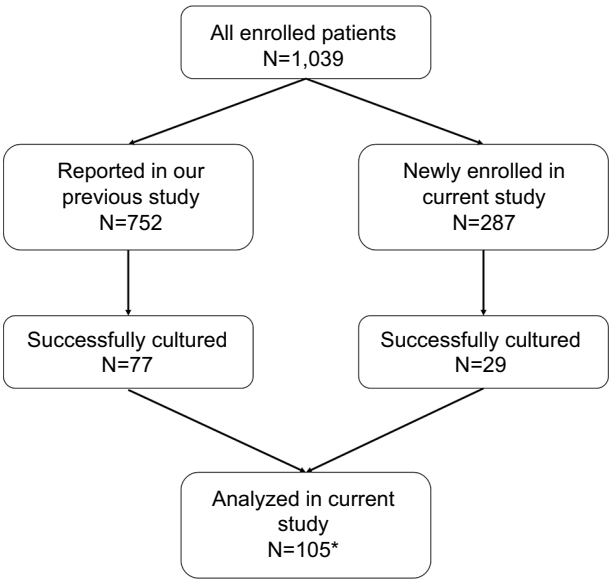

Figure 1 The chart showing the enrollment of patients in the current study.
Note: *One strain (Malang1) could not grow well, and hence, we excluded it from the study.

All procedures applied in this work complied with the ethical standards of the relevant national and institutional committees on human experimentation and with the Declaration of Helsinki of 1975, as revised in 2008 and 2013. Peptic ulcer disease was diagnosed by endoscopic examination, whereas the diagnosis of gastritis was based on histologic examination. The review board or the ethics committee of the following institutions reviewed and approved the study protocol: Dr. Cipto Mangunkusumo Teaching Hospital (Jakarta, Indonesia), Dr. Soetomo Teaching Hospital (Surabaya, Indonesia), Dr. Wahidin Sudirohusodo Teaching Hospital (Makassar, Indonesia), and Oita University Faculty of Medicine (Yufu, Japan). All study participants agreed to follow the study protocol and provided written informed consent. For the participants who were <18 years old, the parents or legal guardian provided written informed consent.

H. pylori was isolated from homogenized antral biopsy specimens by inoculating onto selective agar plates and incubating the plates for up to 10 days in microaerophilic environment (10% O_2, 5% CO_2, and 85% N_2) at 37°C. The colonies that grew were subcultured onto antibiotic-free Mueller–Hinton II agar (Beckton Dickinson, Franklin Lakes, NJ, USA) supplemented with 10% horse blood under the same microaerophilic conditions. *H. pylori* isolates were confirmed based on colony morphology and Gram staining as well as oxidase, catalase, and urease test results. The isolates were stored in Brucella broth (Difco, Franklin Lakes, NJ, USA) supplemented with 10% dimethyl sulfoxide and 10% horse serum at −80°C.

Antibiotic susceptibility testing

The twofold agar dilution method was used to determine minimum inhibitory concentrations (MICs) of furazolidone (Tokyo Chemical Company, Tokyo, Japan), rifaximin (Tokyo Chemical Company), rifabutin (Sigma-Aldrich Co., St. Louis, MO, USA), garenoxacin (Sigma-Aldrich Co.), and sitafloxacin (Haoyuan Chemexpress, Shanghai, China) according to M07-A9 version of methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically (approved standard, Clinical and Laboratory Standard Institute). Briefly, the isolates were subcultured on Mueller–Hinton II agar supplemented with 10% horse blood. The bacteria were diluted in Brucella broth and adjusted to be equivalent to a McFarland opacity standard of 0.5. The prepared bacterial suspension (1 μL) was then inoculated using 2 mm-pin inoculator (Tokken Inc. Chiba, Japan) on Mueller–Hinton II agar supplemented with 5% horse blood. The MICs were determined after a 72-hour incubation. An *H. pylori* strain from American Type Culture Collection (catalog # 43504) was used as the quality control. Resistance breakpoints were determined based on an MIC of >4 mg/L for furazolidone and rifaximin and >1 mg/L for rifabutin, garenoxacin, and sitafloxacin, as described previously.[32–35] The final concentrations of furazolidone and rifaximin ranged from 0.25 to 32 μg/mL, while those of rifabutin, garenoxacin, and sitafloxacin ranged from 0.064 to 8 μg/mL.

Detection of virulence factors and resistant strains

H. pylori DNA was extracted using the commercially available DNeasy® kit (Qiagen, Hilden, Germany) and stored at −20°C until further analysis. Data on the *gyrA* and *gyrB* mutations in *H. pylori* were available for the 752 patients who were reported in our previous publication.[18] In addition, mutation analyses were performed for *gyrA* and *gyrB* mutation status in the remaining 287 specimens. Furthermore, next-generation sequencing (MiSeq next-generation sequencer; Illumina, San Diego, CA, USA) was used to analyze all specimens for full-length *rpoB*, *oipA* status ("on" or "off"), and the presence of *vacA* (s1 or s2; m1 or m2; and i1, i2, or i3), *iceA* (*iceA1* or *iceA2*), *jhp0562*, and *β-(1,3)galT* genotypes of the Indonesian strains. The BLAST algorithm implemented in the CLC Genomics Workbench software (ver. 11; Qiagen NV, Venlo, Netherlands) was used for the analysis. The sequences of hp0701, hp0501, hp1198, and hp0638 of the strain 26695 (GenBank accession number AE000511.1) were used as queries to obtain the *gyrA*, *gyrB*, *rpoB*, and *oipA* sequences, respectively, from the Indonesian next-generation sequencing data. The variants related to antibiotic resistance were predicted by comparing all the *rpoB* sequences of resistant strains and five random sensitive strains with the *rpoB* sequence of the strain 26695 for rifaximin resistance and *gyrA* and *gyrB* for garenoxacin and sitafloxacin resistance. Briefly, after obtaining the *rpoB*, *gyrA,* and *gyrB* sequences and confirming the absence of insertions or deletions leading to frameshift mutations, the sequences were aligned at the codon level using the MAFFT software (http://mafft.cbrc.jp/alignment/server/). Subsequently, each codon of the resistant and sensitive strains was compared 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.

Statistical analyses

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

Results

Resistance of *H. pylori* to alternative antibiotics

Twenty-nine *H. pylori* strains were isolated from 287 patients including 1, 1, 10, 1, 7, and 9 strains from Surabaya, Palembang, Dolok Sanggul, Gunungsitoli, Kolaka, and Merauke, respectively. No *H. pylori* strains could be isolated from the specimens obtained from the patients in Cimacan and Padang. We also reanalyzed the 77 strains that were assessed for their sensitivity to clarithromycin, amoxicillin, metronidazole, tetracycline, and levofloxacin in our previous study.[18] However, one of these strains (Malang1) did not grow properly. Therefore, a total of 105 strains were analyzed in the current study.

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

Rates of antibiotic resistance according to location and ethnicity

The rate of garenoxacin resistance was highest among the *H. pylori* strains obtained from Java Island compared to those from the other regions (15.4% vs 10.0%, 6.2%, and 4.7% from the Sumatera, Papua, and Sulawesi Island, respectively; Table 2). The garenoxacin resistance was not detected in any of the strains from Kalimantan, Timor, and Bali. In contrast, more than half of the strains isolated from the specimens of patients from Kalimantan, Sulawesi, and Bali Islands had rifaximin resistance (60.0%, 52.4%, and 50.0%, respectively). Finally, the rate of rifaximin resistance was at least 20% in all the study locations.

The analysis of the rates of antibiotic resistance according to ethnicity revealed that the garenoxacin resistance rate of 20% was higher in the strains isolated from the Chinese Indonesian patients than in those isolated from the Bataknese, Buginese, and Papuan patients (9.6%, 7.7%, and 6.2%, respectively; Table 3); however, this difference was not statistically significant (*P*=0.44, *P*=0.33, and *P*=0.31, respectively). None of the

Table 1 Rates of resistance to alternative antibiotics in *Helicobacter pylori* strains isolated in Indonesia

Characteristics	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)

Abbreviation: PUD, peptic ulcer disease.

Table 2 Rates of resistance to alternative antibiotics in *Helicobacter pylori* strains isolated in 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[a]	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)

Note: [a]Strains obtained from patients from Nias Island were combined with those of Sumatera Island due to the low sample number.

Table 3 Prevalence of antibiotic resistance in *Helicobacter pylori* isolates based on ethnicity

Ethnicity	Island	N	Resistance (%)				
			FUR	SIT	GAR	RFX	RIF
Ambonese	Java	4	0 (0.0)	0 (0.0)	0 (0.0)	2 (50.0)	0 (0.0)
Bataknese	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)

Abbreviations: FUR, furazolidone; GAR, garenoxacin; RFX, rifaximin; RIF, rifabutin; SIT, sitafloxacin.

strains isolated from the Ambonese, Balinese, Dayak, Javanese, Minahasanese, and Timor patients exhibited garenoxacin resistance, indicating that the bacterial strains in the patients belonging to these ethnic groups were sensitive to furazolidone, rifabutin, garenoxacin, and sitafloxacin but not rifaximin. Only one strain from a Javanese patient (only one strain was isolated) was sensitive to all five antibiotics. Rifaximin resistance was distributed evenly among ethnic groups (Table 3), with the rates ranging between 25.0% and 69.2% (P=0.81). Within the ethnic groups with the highest prevalence of *H. pylori* in Indonesia,[19] all the strains were resistant to garenoxacin and rifaximin, with the higher resistance rate to rifaximin observed in the Buginese patients compared with that in the Papuan and Bataknese patients (9/13 [69.2%] vs 6/16 [37.5%], P=0.01 and 8/31 [25.8%], P=0.001, respectively).

Comparison of alternative and standard antibiotic regimens

We determined the rates of resistance of the 76 *H. pylori* strains reported in our previous study[14] to the five antibiotics and compared them with the rates of resistance to the standard antibiotics used for *H. pylori* infection (Table 4). Our findings above indicated that, except for rifaximin with a resistance rate of 35.5% (27/76), there was a possibility that the remaining four antibiotics might overcome the high rate of resistance to levofloxacin and metronidazole. Interestingly, *H. pylori* in the regions with high rates of clarithromycin resistance, such as Bali and Papua Islands (1/6, 16.7% and 1/7, 14.3%, respectively), was still sensitive to garenoxacin, although this finding could be due to the low number of strains with clarithromycin resistance. In contrast, the isolate from Java Island with the highest rate of clarithromycin resistance also exhibited a high rate of rifaximin resistance. Thus, we suggest that rifaximin should not be considered as an alternative in areas with high clarithromycin resistance. Garenoxacin may combat *H. pylori* in regions with high amoxicillin resistance such as Papua Island (0.0% vs 14.3% of resistance rate for garenoxacin and amoxicillin, respectively) but not in regions with high tetracycline resistance such as Java Island (both 15.4% of resistance rate) (Table 4).

To further analyze the associations among resistance rates of metronidazole, levofloxacin, garenoxacin, and rifaximin, we created a two-by-two table (Figure 2). Only seven strains (9.2%) exhibited resistance to both rifaximin and levofloxacin.

Table 4 Comparison of the five alternative antibiotics with the standard regimens as reported by Miftahussurur et al[18]

Island	n	Resistant regimens (%)									
		CAM	AMX	MNZ	TCN	LVX	FUR	SIT	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)

Abbreviations: AMX, amoxicillin; CAM, clarithromycin; FUR, furazolidone; GAR, garenoxacin; LVX, levofloxacin; MNZ, metronidazole; RFX, rifaximin; RIF, rifabutin; SIT, sitafloxacin; TCN, tetracycline.

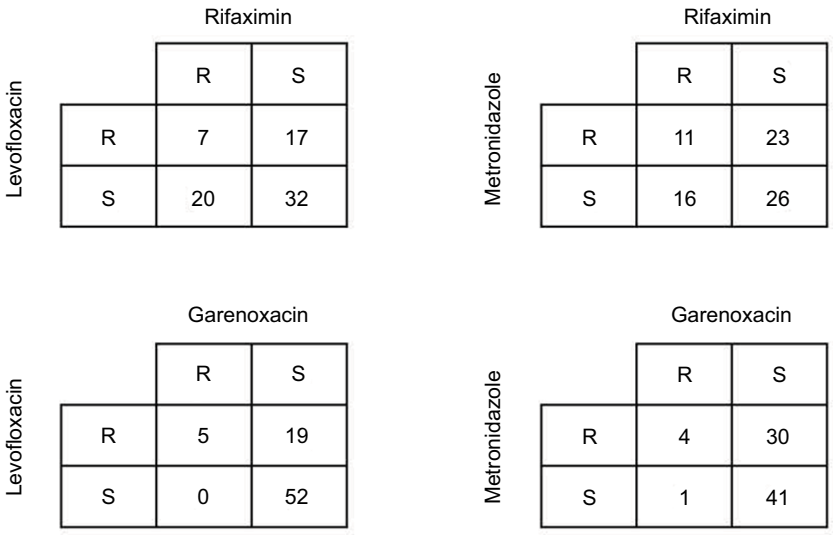

Figure 2 The associations among rates of resistance to metronidazole, levofloxacin, garenoxacin, and rifaximin.

Furthermore, the percentage of the levofloxacin-resistant/rifaximin-sensitive strains was lower than that of the levofloxacin-sensitive/rifaximin-resistant strains (17/76 [22.4%] vs 20/76 [26.3%]). In contrast, the percentage of the metronidazole-resistant/rifaximin-sensitive strains was higher than that of the metronidazole-sensitive/rifaximin-resistant strains (23/76 [30.3%] vs 16/76 [21.1%]). Conversely, the percentages of the metronidazole-resistant/garenoxacin-sensitive and the levofloxacin-resistant/garenoxacin-sensitive strains were higher than those of the metronidazole-sensitive/garenoxacin-resistant and levofloxacin-sensitive/garenoxacin-resistant strains (19/76 [25.0%] and 30/76 [39.5%] vs 0/76 [0.0%] and 1/76 [1.3%], respectively).

Mutations associated with garenoxacin resistance

We analyzed the *gyrA* and *gyrB* mutations from the two strains with high MICs for garenoxacin identified in the current study (Merauke20 and Kolaka72; Table 5) together with those identified in our previous study.[18] Four strains with the highest MICs for garenoxacin (2 mg/L) were associated with a high MIC for levofloxacin (>32 mg/L). In addition, three of these strains had an amino acid substitution at Asp91 or Asn87 in the GyrA, which were predominantly associated with the highest MICs for levofloxacin.[18] However, the 13 garenoxacin-sensitive strains with low MIC values (<0.063–0.5 mg/L) were also associated with those mutations, suggesting an inconsistent effect of these mutations. Moreover, none of the garenoxacin-resistant strains harbored a substitution at Arg484 or Ser479 of the *gyrB*. Finally, none of the strains harbored *parC* or *parE*, the two important genes associated with quinolone resistance in other bacteria.

Mutations associated with rifaximin resistance

We analyzed full-length *rpoB* from 40 rifaximin-resistant strains based on the next-generation sequencing data, with an

Table 5 Mutations associated with quinolones

No.	Strains	*gyrA* mutation	*gyrB* mutation	MIC of LVX (mg/L)	MIC of GAR (mg/L)	MIC of 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[a]	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	Medan10	None	None	25	<0.063	<0.063
12	Medan15	R140K, D192N	None	>32	2	0.5
13	Medan17	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	Medan23	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[b]	E103G	None	na	1	<0.063
26	Kolaka72[b]	D91G	None	na	1	<0.063

Notes: An MIC >1 mg/L was used as a resistance breakpoint for levofloxacin, garenoxacin, and sitafloxacin. [a]One of the previously isolated strains could not sustain growth. [b]The new strains with high MIC of garenoxacin that were not reported in our previous study.
Abbreviation: GAR, garenoxacin; LVX, levofloxacin; MIC, minimum inhibitory concentration; na, not available; SIT, sitafloxacin.

average sequencing coverage ranging from 82.43x to 560.85x and a Q_{30} score percentage ranging from 80.59% to 96.31% (Table S1). Five random rifaximin-sensitive strains were used for comparison. Pairwise alignment identified that the garenoxacin-sensitive strains shared 95.7%–97.8% identity with the reference strain 26695. Using the strain 26695 and the garenoxacin-sensitive control strains, DNA sequence analysis of *rpoB* from all rifaximin-sensitive strains revealed intact reading frames that lacked nonsense mutations. Among all 2,890 codons of *rpoB*, 1,010 had non-synonymous substitutions, indicating a change of nucleotide without a change in the amino acid (silent mutations). In contrast, majority of the rifaximin-resistant strains (39/40 [97.5%]) contained missense mutations (Table 6). We confirmed that the predominant point mutations of *rpoB* were the replacement of isoleucine at position 837 with valine amino acid (8/40 [20%]), alanine at position 2,414 with valine or threonine (8/40 [20%]), glutamine at position 2,079 with lysine (7/40 [17.5%]), and lysine at position 2,068 with arginine (7/40 [17.5%]).

Virulence factors and antibiotic resistance types

In addition to the data on virulence factors that we reported previously,[18] we analyzed virulence factors in the 29 newly identified *H. pylori* strains including *cagA*, *vacA*, *iceA*, *jhp0562*/*β-(1,3)galT*, and *oipA*. There was an association between the *vacA* genotype of *H. pylori* with rifaximin resistance (P=0.048). The genotypes s2m1 and s1m1 of *vacA* tended to be more frequent in the garenoxacin-resistant strains compared with the *vacA* s1m2 and s2m2 genotypes (2/2 [100.0%], 32/74 [43.2%], 6/26 [23.1%], and 0/2 [0.0%], respectively; P=0.051). There were no significant associations between other virulence factors and antibiotic resistance.

Nucleotide sequencing

The nucleotide sequences were deposited in the DDBJ under accession numbers LC420353–LC420380 (*vacA*), LC420381–LC420408 (*oipA*), LC420409–LC420436 (*jhp0562* and *jhp0563*), LC420437–LC420462 (*iceA*),

Table 6 Mutations associated with rifaximin resistance

No.	Strain name	MIC (mg/L)	*rpoB* 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, A735T, 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, L2881I
40	Medan25	8	V52I, I66V, E106G, V657I, A756V, D2380E, K2482R

Note: S355Y means tyrosine replaced serine amino acid in the position 355.
Abbreviation: MIC, minimal inhibitory concentration.

LC420463–LC420466 (*gyrA* and *gyrB*), and LC420467–LC420511 (*rpoB*).

Discussion

The current study revealed that none of the *H. pylori* strains isolated from Indonesian patients were resistant to furazolidone, suggesting that furazolidone might be considered as an alternative *H. pylori* treatment regimen in Indonesia, especially in regions with high prevalence of strains exhibiting dual resistance to clarithromycin and metronidazole.[36] Our results are in agreement with those reported by a study from a neighboring country, Malaysia,

which also found that all the isolated strains were sensitive to furazolidone.[37] Furazolidone use has been proposed in recent guidelines for *H. pylori* management in developing countries, due to its efficacy, low rate of primary bacterial resistance, and lack of alternative and low-cost therapies.[38,39] To improve the *H. pylori* cure rates, bismuth should be added to therapy. For example, the addition of bismuth to quadruple therapy including furazolidone has been successful in China, with cure rates reaching 92.26% with minimal side effects.[40] However, bismuth is unavailable in certain regions[41] due to potential bismuth-associated carcinogenic effects, including mutagenicity and genotoxicity in in vitro and animal models,[42,43] and it was classified as a type III carcinogen for humans in 1997 by the International Agency on Research on Cancer. Furthermore, there are currently no standardized rescue therapies available for patients who fail the initial furazolidone-based treatment.

Our finding of all isolated strains exhibiting rifabutin sensitivity provides support for rifabutin as a potential alternative antibiotic against *H. pylori*. The concentrations of rifabutin in the gastric juice were reported to be 10–17 times higher than in peripheral blood.[44] The antibacterial activity of rifabutin, which is not affected by the low pH environment in the stomach, is higher than that of rifampicin.[27] Importantly, its target is different from that of clarithromycin. Therefore, its efficacy in strains with primary clarithromycin resistance, even in those who are also resistant to metronidazole, is high,[45,46] although the majority of the clinical trials that define these differences were conducted in Western countries. Nonetheless, the adverse effects of rifabutin such as myelotoxicity should be considered. Furthermore, the increased use of rifabutin in Indonesia, a country with high tuberculosis prevalence, might lead to rifabutin resistance of *Mycobacterium tuberculosis*. Several studies reported substantial in vitro cross-resistance to rifampicin, a main component of the tuberculosis therapy regimens, although rifabutin resistance in *H. pylori* in *in vitro* was rarely reported.[28] A history of rifampicin treatment should be taken into consideration before prescribing rifabutin for *H. pylori* eradication to reduce the possibility of failure of tuberculosis treatment and *H. pylori* eradication. Importantly, rifabutin use in combination with clarithromycin should be avoided, based on evidence showing the inhibition of rifabutin metabolism by clarithromycin in liver microsomes,[47] which suggests that potential toxicity might arise with combination use.

Compared with the other fluoroquinolones, sitafloxacin is a more potent inhibitor of DNA gyrase and topoisomerase IV, which play important roles in bacterial DNA repair, transcription, replication, and recombination.[48] Sitafloxacin improves the efficacy of quinolone-based rescue therapy by virtue of its ability to eradicate *H. pylori* strains with *gyrA* mutations.[49] However, limited access and availability are the main concerns regarding sitafloxacin. Currently, Japan and Thailand are the only countries that provide sitafloxacin in their health care system, and clinical trials for sitafloxacin are underway in Western countries.[50] In contrast, although garenoxacin was also reported to eradicate *H. pylori* strains with *gyrA* mutations,[51] there were several strains that were resistant to this antibiotic in the current study. Interestingly, the MIC value of levofloxacin was not associated with MIC value of garenoxacin. For example, although all seven garenoxacin-resistant strains exhibited the highest MICs for levofloxacin (>32 mg/L), all ten strains with the highest levofloxacin MIC were sensitive to garenoxacin. The lower antibacterial activity of garenoxacin against *H. pylori* compared with that of sitafloxacin might be associated with the high affinity of sitafloxacin to DNA gyrase.[52] Due to high levofloxacin resistance rates in Indonesia, our finding should be instrumental in formulating second-line regimen guidelines to eradicate *H. pylori*.

One study found that double mutations in *gyrA* were associated with a sevenfold increase in sitafloxacin MIC compared with the pretreatment MICs and that double mutations in *gyrA*, including the mutations at Asp91 and Asn87, were associated with eradication failure.[53] We found that double mutations were not associated with an increase in the MIC of sitafloxacin, although none of the strains harbored both Asp91 and Asn87 mutations. Similar to sitafloxacin, none of the single or double mutations in GyrA or GyrB were associated with garenoxacin resistance. Although *parC* and *parE* are important genes associated with quinolone resistance, none of the isolated *H. pylori* strains exhibited the presence of/expressed these genes, as previously described.[54] Our results suggest that genes other than *gyrA* or *gyrB* were associated with resistance to sitafloxacin and garenoxacin, which should be investigated in future studies.

Among the several alternative drugs tested in the current study, the rate of resistance was highest to rifaximin; this finding is in agreement with a previous study showing that rifaximin-based triple therapy did not achieve acceptable *H. pylori* cure rates.[55,56] However, rifaximin is a promising *H. pylori* drug due to poor absorbance in the blood, which can minimize adverse effects, and its higher bioavailability in the gastrointestinal tract than that of other antibiotics.[57] The poor eradication rates might be due to a failure in achieving sufficient therapeutic concentrations under and within the

gastric mucosal layer, which is a frequent site of *H. pylori* colonization.[58] Therefore, well-designed clinical trials are necessary to evaluate rifaximin efficacy against *H. pylori*, including high-dose regimens of longer duration, additional bioadhesive formulations, and combinations with mucolytic agents for persistent coverage of the gastric mucosa.[58]

Although *rpoB* mutations were reported to play a role in rifaximin resistance in other bacteria such as *Escherichia coli*,[59] *Clostridium difficile*,[60,61] *Staphylococcus*,[62] and *M. tuberculosis*,[63] only one study found an association between codons 524–545 and 585 of *rpoB* with rifabutin resistance in *H. pylori*.[30] In the current study, we found numerous missense mutations in *rpoB* of rifaximin-resistant strains, including novel and predominant mutations: 837I, 2414A, 2079K, and 2068K. Although the mechanism remains unclear, the risk for horizontal transmission of the *rpoB* mutations is lower than that of a resistance gene located on a plasmid or transposon; however, certain yet-to-be determined conditions and improper prescription or usage of antibiotic could still facilitate the rapid transmission of such mutations.[62] Strict control should be practiced to prevent rifaximin failure in *H. pylori* eradication.

The major limitation of this study was the relatively small number of samples, and when we divided the samples based on regions, it yielded very low sample number in each region. Therefore, it may be difficult to represent *H. pylori* strains in whole Indonesia. Further study with a bigger sample size is necessary. However, these samples, obtained from 1,039 endoscopic patients, comprised the biggest cohort of *H. pylori* strains isolated in Indonesia thus far. Indonesia is a wide country and consists of many ethnic groups. Among those, some ethnic groups had a much higher prevalence of *H. pylori* infection than the others; however, the overall gastric cancer risk in Indonesia is low, suggesting that Indonesia may become the best example for Asian enigma similar to South Asia. In addition, only a fraction of the genomic changes that were related to drug resistance, among a total of 1,600 genes of *H. pylori*, were examined in the current study. Although sitafloxacin is a potent drug for *H. pylori*, it has not been approved by the Indonesian National Agency of Drug and Food Control. Thus, sitafloxacin-based regimens cannot be currently prescribed in Indonesia.

Conclusion

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

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Author contributions

All authors contributed toward data analysis, drafting and revising the paper, gave final approval of the version to be published and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Mutations associated with rifaximin resistance

No.	Strain ID	Average coverage	Q_{30} percentage	Rifaximin
1	Manado1	326.27	91.21	S
2	Surabaya71	346.57	90.99	S
3	Surabaya47	264.51	88.23	R
4	Surabaya68	408.21	91.31	S
5	Surabaya69	504.26	88.53	S
6	Surabaya71	560.21	88.06	S
7	Surabaya79	554.35	94.39	S
8	Jayapura1	570.45	95.95	S
9	Jayapura3	560.85	96.31	S
10	Jayapura6	589.56	95.97	R
11	Jayapura8	231.21	84.54	S
12	Jayapura15	489.67	96.36	S
13	Jayapura16	509.21	95.14	S
14	Jayapura21	502.21	96.49	R
15	Jakarta9	115.74	91.23	R
16	Medan17	142.34	80.59	S
17	Medan23	166.24	90.67	S
18	Medan27	91.58	80.76	S
19	Medan3	93.57	80.88	S
20	Medan10	85.96	80.44	S
21	Medan11	278.2	87.68	S
22	Medan15	260.45	80.59	R
23	Medan19	176.28	83.32	S
24	Medan20	164.28	82.27	S
25	Medan22	115.51	82.74	R
26	Medan23	264.07	81.06	S
27	Medan25	219.52	80.97	R
28	Medan28	169.54	80.8	S
29	Medan30	237.04	80.42	S
30	Makasar31	87.65	81.19	R
31	Makasar45	156.35	89.51	R
32	Makasar47	85.35	80.68	S
33	Makasar52	87.07	83.13	R
34	Makasar55	89.29	83.49	R
35	Makasar56	82.43	82.46	S
36	Pontianak63	82.77	88.97	S
37	Pontianak75	85.13	86.89	R
38	Pontianak20	191.58	89.57	S
39	Pontianak44	128.24	89.45	R
40	Pontianak50	99.51	91.01	R
41	Manado5	171.08	89.76	S
42	Manado18	136.04	90.18	S
43	Manado20	110.47	87.37	S
44	Manado26	151.85	86.67	S
45	Manado28	175.98	93.44	S
46	Manado29	90.53	93.63	R
47	Manado31	102.87	93.18	R
48	Kupang2	156.23	82.79	S
49	Kupang5	132.2	85.25	S
50	Kupang6	120.2	83.27	S
51	Kupang10	105.54	82.16	R
52	Kupang11	196.38	80.2	S
53	Kupang15	192.96	82.36	S
54	Kupang23	125.05	84.94	R
55	Kupang26	98.87	89.29	R
56	Kupang28	146.88	86.58	S
57	Kupang29	144.7	81.55	R
58	Kupang30	85.85	85.47	R
59	Kupang33	158.02	83.55	S
60	Kupang34	238.16	87.82	R
61	Kupang35	240.73	85.3	S
62	Kupang41	112.69	85.57	S
63	Kupang42	86.98	81.43	R
64	Kupang47	142.12	85.5	R
65	Kupang64	171.52	82.59	R
66	Kupang73	134.33	88.07	S
67	Kupang83	152.03	85.08	S
68	Medan18	201.24	89.67	S
69	Medan31	162.6	90.39	S
70	Medan32	264.33	88.11	S
71	Medan33	368.99	86.63	S
72	Nias9	123.05	84.92	S
73	Medan36	97.32	84.74	S
74	Medan37	95.96	85.43	S
75	Medan40	125.98	85.11	S
76	Medan49	163.37	83.54	S
77	Medan50	146.38	83.51	S
78	Medan56	192.02	83.51	R
79	Medan67	223.14	85.69	R
80	Medan68	137.93	84.11	S
81	Medan73	224.19	83.87	S
82	Medan75	194.47	83.86	R
83	Padang42	157.75	88.29	R
84	Surabaya106	84.06	80.06	S
85	Surabaya137	104.09	86.49	S
86	Surabaya151	96.79	82.68	S
87	Surabaya192	106.55	85.02	S
88	Surabaya283	107.28	87.04	R
89	Surabaya304	112.26	86.21	R
90	Merauke3	243.44	85.05	S
91	Merauke5	219.32	84.41	S
92	Merauke7	225.09	83.16	S
93	Merauke8	269.32	86.05	S
94	Merauke12	191.03	85.7	S
95	Merauke20	145.23	88.24	R
96	Merauke21	338.84	87.53	R
97	Merauke27	185.99	80.84	R
98	Merauke37	304.88	86.56	R
99	Kolaka56	219.48	85.48	S
100	Kolaka72	196.53	86.86	R
101	Kolaka79	343.49	86.15	R
102	Kolaka94	256.4	83.82	S
103	Kolaka96	190.85	82.58	R
104	Kolaka98	206.04	83.99	R
105	Kolaka99	279.25	81.86	R

Abbreviations: R, resistant; S, sensitive.