

RESEARCH ARTICLE

Surveillance of *Helicobacter pylori* Antibiotic Susceptibility in Indonesia: Different Resistance Types among Regions and with Novel Genetic Mutations

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Abstract

Information regarding *Helicobacter pylori* antibiotic resistance in Indonesia was previously inadequate. We assessed antibiotic susceptibility for *H. pylori* in Indonesia, and determined the association between virulence genes or genetic mutations and antibiotic resistance. We recruited 849 dyspeptic patients who underwent endoscopy in 11 cities in Indonesia. E-test was used to determine the minimum inhibitory concentration of five antibiotics. PCR-based sequencing assessed mutations in *23S rRNA*, *rdxA*, *gyrA*, *gyrB*, and virulence genes. Next generation sequencing was used to obtain full-length sequences of *23S rRNA*, *infB*, and *rpl22*. We cultured 77 strains and identified 9.1% with clarithromycin resistance. Low prevalence was also found for amoxicillin and tetracycline resistance (5.2% and 2.6%, respectively). In contrast, high resistance rates to metronidazole (46.7%) and levofloxacin (31.2%) were demonstrated. Strains isolated from Sumatera Island had significantly higher metronidazole resistance than those from other locations. Metronidazole resistant strains had highly

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distributed *rdxA* amino acid substitutions and the *23S rRNA* A2143G mutation was associated with clarithromycin resistance (42.9%). However, one strain with the highest MIC value had a novel mutation in *rpl22* without an A2143G mutation. Mutation at Asn-87 and/or Asp-91 of *gyrA* was associated with levofloxacin-resistance and was related to *gyrB* mutations. In conclusions, although this is a pilot study for a larger survey, our current data show that Indonesian strains had the high prevalence of metronidazole and levofloxacin resistance with low prevalence of clarithromycin, amoxicillin, and tetracycline resistance. Nevertheless, clarithromycin- or metronidazole-based triple therapy should be administered with caution in some regions of Indonesia.

Introduction

Asia is a very important continent for *Helicobacter pylori* infection, a common chronic bacterial infection in humans that is associated with peptic ulcer disease, gastric cancer, and primary gastric B-cell lymphoma [1]. Asia is the continent with the largest populace (4.4 billion people) and the highest frequency of gastric cancer in the world [2]. With some exceptions that some population with low incidence of gastric cancer even with a high prevalence of *H. pylori* known as the “Asian enigma” [3], the incidence of gastric cancer in several regions tends to mirror the prevalence of *H. pylori* infection [4]. Recent guidelines have proposed indications and regimens for the Asia-Pacific region and three countries in East Asia [5–8]. Nevertheless, the development of drug resistance in *H. pylori* is a major issue and the viability of some regimens has been truly challenged as they are becoming unsuccessful [9]. Creating an updated suitable first-line regimen is fundamental to counteract revised treatment courses that result in perpetuation of secondary antibiotic resistance [2].

Indonesia is a multi-ethnic nation in Southeast Asia that consists of more than 13,600 islands, with Sumatra, Papua, Kalimantan, Sulawesi, and Java being the five main islands. Currently, hospitals that provide GI endoscopy services in Indonesia are limited, and most are located on Java Island [10]. The prevalence of *H. pylori* infection in Javanese, the predominant ethnicity, is low (2.4%), even when using a combination of five different diagnostic methods [11,12]. However, several ethnic groups have much higher prevalence of *H. pylori* infection (42.9%, 40.0%, and 36.7% for Papuan, Batak, and Buginese individuals, respectively) [12]. Additionally, they harbor more virulent genotypes such as *cagA* positive, *oipA* ‘on’, and *iceA1* positive [13]. The Kyoto Global Consensus Conference on *H. pylori* Gastritis in 2014 expressed that *H. pylori* gastritis should be characterized as an infectious disease, notwithstanding asymptomatic patients and irrespective of complications such as peptic ulcers and gastric cancer [14]. It has been proposed that *H. pylori* should be eradicated, even in Indonesia. To our knowledge, only a single study has reported the rates of *H. pylori* antibiotic resistance in Indonesia, identifying 72 strains in the low prevalence *H. pylori* region, Jakarta, in 2006 [15]. In this study, the rates clarithromycin (CAM), amoxicillin (AMX), metronidazole (MNZ), and levofloxacin (LVX) resistance were 27.8%, 19.4%, 100.0%, and 1.4%, respectively [15]. In addition, there was no report regarding tetracycline (TCN) resistance in Indonesian in *H. pylori* strains. TCN is a basic antibiotic, used in quadruple regimens, for *H. pylori* eradication. Since antibiotic resistance is expanding globally [16,17], it is necessary to examine the drug resistance rates in Indonesia.

The understanding of *H. pylori* antibiotic resistance mechanisms, which primarily occur because of mutations in chromosomal genes, is important as a premise for the establishment

of rational antibiotic combinations. Critically, although numerous point mutations have appeared, the positions of such mutations were not uniform for every topographical area [18,19]. For example, although two nucleotide substitutions, specifically, A2142G or A2142C and A2143G in the peptidyl transferase loop of 23S *rRNA*, cause primary CAM resistance in *H. pylori* strains isolated in Western countries [20], they account for only 23% of resistant strains in Asia [20]. Our previous report demonstrated the synergic effect of mutated sequences in *hp1048* (*infB*), *hp1314* (*rpl22*), and A2143G, which resulted in higher MICs [21]. Unlike the mutational patterns of 23S *rRNA*, the mechanisms of MNZ resistance are complex and largely associated with inactivating mutations in *rdxA*, through frameshift mutations, insertions, and deletions [22]. Moreover mutations in the sequences of gyrase subunit A (*gyrA*) and *gyrB*, encoding translational proteins, greatly reduce the antimicrobial ability of fluoroquinolones [23].

In this study, we aimed to determine the antibiotic susceptibility of *H. pylori* in 11 cities, covering the five largest islands, of Indonesia. We also analyzed the association between virulence genes and antibiotic resistance rates. Furthermore, we determined the presence of genetic mutations that are associated with antibiotic resistance.

Materials and Methods

Patients and *H. pylori*

We conducted a prospective study from August 2012 to November 2015. This study included 849 adult dyspeptic patients who underwent endoscopy examinations in 11 cities including cities on the five largest islands of Indonesia. Patients with bleeding related to esophageal varices, a history of partial gastric resection and previous *H. pylori* eradication therapy were excluded from this study. Included were patients from Surabaya (296 patients), Jakarta (31), and Malang (97) on Java Island, Aceh (38) and Medan (93) on Sumatera Island, Pontianak (90) on Kalimantan Island, Makassar (30) and Manado (57) on Sulawesi Island, Jayapura (21) on Papua Island, Bangli (61) on Bali Island, and Kupang (35) on Timor Island. Peptic ulcer diseases were diagnosed by endoscopic observation, whereas chronic gastritis was determined by histologic examination. All procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. There were no minors or children enrolled in our study, therefore not needed informed consent from the next of kin, care-takers, or guardians on behalf of them. Written informed consent was obtained from all participants, and the study protocol was approved by the Institutional Review Board or the Ethics Committee of 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).

For *H. pylori* culture, antral biopsy specimens were homogenized and inoculated onto antibiotics selection plate, subsequently subcultured onto Mueller Hinton II Agar medium (Becton Dickinson, NJ, USA) supplemented with 10% 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

The E-test method (Biomerieux, 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 at <http://www.eucast.org/>). Strains were considered to be resistant for MICs of >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.

Virulence factors and resistant strains molecular detection

H. pylori DNA was extracted from *H. pylori*, cultured to confluence on plates, using a commercially available kit (Qiagen, Hilden, Germany). The DNA was stored at -20°C until used for gene amplification. Mutations in *gyrA*, *gyrB*, *rdxA*, and 23S *rRNA* were assessed in antibiotic-resistant strains by PCR-based sequencing using the primers as described previously [24–26]. As a control, we sequenced five randomly selected strains sensitive to MNZ and LVX and one CAM-sensitive strain from the collection of Indonesian strains. The sequences were then compared with the published sequence of *H. pylori* strain 26695 (GenBank accession number AE000511.1 GI: 6626253) using MAFFT version 7 (available at <http://mafft.cbrc.jp/alignment/server/>) and confirmed by visual inspection. Direct sequencing of the *cagA* EPIYA repeat region and determination of *oipA* status (“on” or “off”) were determined by PCR-based sequencing as described previously [27–29]. The presence of *vacA* (s1 or s2, m1 or m2 and i1, i2 or i3), *iceA* (*iceA1* or *iceA2*), *dupA*, *jhp0562*, and β -(1,3)*galT* genotypes were determined based on PCR product size as described previously [30–35].

To find other genetic mutations, associated with high MIC values, but not involving typical 23S *rRNA* mutations, we also obtained full-length 23S *rRNA*, *hp1048* (*infB*), and *hp1314* (*rpl22*) [21] from next-generation sequencing 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, whereas continuous variables were tested using the Mann-Whitney *U* and *t*-tests. A multivariate logistic regression model was used to calculate the odds ratios (OR) of clinical outcomes including age, sex, *H. pylori* antibiotic resistance, ethnicity, and location. All determinants with *P* values < 0.10 were entered together into the full logistic regression model. The OR and 95% confidence interval (CI) were used to estimate risk. A *P* value of < 0.05 was accepted as 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

Despite obtaining a large amount of samples, only 77 strains could be isolated. The low number of isolated strains was related to the low prevalence of *H. pylori* infection in Indonesia, and was confirmed by histology and immunohistochemistry (88/849, 10.4%, Table 1). Half of the samples were obtained from Java Island (424 samples) and had a *H. pylori* infection prevalence of only 4.0% (17/424; Table 1). In contrast, the most recent survey of Timor Island found the

Table 1. Prevalence of *H. pylori* infection in Indonesia based on multiple tests.

| Island (city) | Year | N | Diagnostic Method (%) | | |
|------------------------|-----------|-----|-----------------------|----------------------------|---------------------|
| | | | Culture | Histology confirmed by IHC | At least one method |
| Bali (Bangli)* | 2015 | 61 | 6 (9.8) | 7 (11.5) | 7 (11.5) |
| Java | | 424 | 14 (3.3) | 15 (3.5)*** | 17 (4.0) |
| (Surabaya) | 2012–2015 | 296 | 12 (4.1) | 14 (4.7) | 15 (5.1) |
| (Jakarta) | 2013 | 31 | 1 (0.1) | 1 (0.1) | 1 (0.1) |
| (Malang)* | 2014 | 97 | 1 (1.0) | ** | 1 (1.0) |
| Kalimantan (Pontianak) | 2014 | 90 | 5 (5.6) | 4 (4.4) | 6 (6.7) |
| Papua (Jayapura) | 2013 | 21 | 9 (42.9) | 9 (42.9) | 9 (42.9) |
| Sumatera | | 131 | 19 (14.5) | 20 (15.3) | 26 (19.8) |
| (Medan) | 2014 | 93 | 19 (20.4) | 20 (21.5) | 26 (27.9) |
| (Aceh)* | 2014 | 38 | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Sulawesi | | 87 | 13 (14.9) | 13 (14.9) | 13 (14.9) |
| (Manado)* | 2015 | 57 | 7 (12.3) | 7 (12.3) | 7 (12.3) |
| (Makassar) | 2014 | 30 | 6 (20.0) | 6 (20.0) | 6 (20.0) |
| Timor (Kupang)* | 2015 | 35 | 14 (40.0) | 12 (34.3) | 14 (40.0) |
| Total | | 849 | 80 (9.4) | 80 (9.4)*** | 88 (10.4) |

IHC: Immunohistochemistry

* The most recent surveys that are not including in the previous publication (Syam AF, *et al.*, 2015)

** Sample obtained only from culture, there was no sample for histology examination

*** The total number does not include the Malang survey

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prevalence of *H. pylori* infection to be 40.0% (14/35). The host individuals consisted of 39 males (age range, 24 to 80 years; mean age, 49.6 ± 10.8 years) and 38 female patients (age range, 28 to 68 years; mean age 48.9 ± 15.4 years). Strains were isolated from Surabaya (12 strains), Jakarta (1), Malang (1), Medan (18), Pontianak (5), Makassar (6), Manado (7), Jayapura (7), Bali (6), and Kupang (14 strains). Among the patients, 70 had chronic gastritis and seven had peptic ulcer diseases.

Overall, there were 28 strains that were sensitive to all antibiotics (36.4%). In contrast with the previous study in Indonesia [15], we found a low prevalence of CAM resistance (7/77, 9.1%, compared to 27.8% in the previous study). Low prevalence was also observed for AMX resistance (4/77, 5.2%) and TCN resistance (2/77, 2.6%, Table 2). In contrast, and in accordance with the trend of increasing resistance Asia [36], there was high rate of resistance to MNZ (36/77, 46.8%). Moreover, most strains were associated with MIC values ≥ 48 mg/L (26/36, 72.2%, Fig 1). In addition, we detected a high prevalence of LVX resistance (24/77, 31.2%) with a high distribution of MIC values that were predominantly ≥ 16 mg/L. The distribution of patient age and antimicrobial resistance of the isolates is shown in Table 2. Antibiotic resistance rate did not differ among different age groups (P = 0.37, 0.42, 0.07, 0.56, and 0.45 for AMX, CAM, MNZ, LVX, and TCN, respectively). There were no associations between antibiotic resistance and gender or clinical outcomes (P > 0.05).

Antibiotic resistance rates according to location

The prevalence of antibiotic resistance based on location is shown in Table 3. Strains from Kalimantan were resistant to only two antibiotics. Strains obtained from Java and Bali Islands had CAM resistance rates greater than 15%, which is the permitted limit of CAM resistance recommended to be used for eradication therapy without checking the resistant rate [37,38].

Table 2. Prevalence of antibiotic resistance of *H. pylori* isolates in Indonesia.

| Characteristic | N | Antibiotics (%) | | | | |
|------------------|----|-----------------|----------|-----------|-----------|----------|
| | | CAM | AMX | MNZ | LVX | TCN |
| Total | 77 | 7 (9.1) | 4 (5.2) | 36 (46.7) | 24 (31.2) | 2 (2.6) |
| Gender | | | | | | |
| Male | 38 | 3 (7.9) | 2 (5.2) | 21 (55.2) | 11 (28.9) | 1 (2.7) |
| Female | 39 | 4 (10.2) | 2 (5.1) | 15 (38.4) | 13 (33.3) | 1 (2.6) |
| Age Groups | | | | | | |
| 17–30 | 9 | 0 (0.0) | 0 (0.0) | 6 (66.6) | 3 (33.3) | 0 (0.0) |
| 31–40 | 10 | 2 (20.0) | 0 (0.0) | 6 (60.0) | 3 (30.0) | 1 (10.0) |
| 41–50 | 21 | 2 (9.1) | 1 (4.7) | 5 (22.7) | 4 (18.1) | 1 (4.5) |
| 51–60 | 25 | 1 (4.1) | 1 (4.0) | 14 (58.3) | 10 (41.6) | 0 (0.0) |
| >61 | 12 | 2 (16.6) | 2 (16.6) | 5 (41.6) | 4 (33.3) | 0 (0.0) |
| Clinical Outcome | | | | | | |
| Gastritis | 70 | 6 (8.9) | 4 (5.9) | 30 (44.7) | 21 (31.3) | 0 (0.0) |
| PUD | 7 | 0 (0.0) | 0 (0.0) | 3 (42.8) | 0 (0.0) | 0 (0.0) |

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

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AMX resistance was not detected in strains obtained from three islands (Bali, Java, and Kalimantan). Only Java Island showed signs of increased resistance to TCN (2/14, 14.3%). The highest LVX resistance was in strains isolated from Java and Sumatera Islands (50.0% and 44.4%, respectively). Strains isolated from Sumatera Island showed significantly higher MNZ resistance (88.9%) than those of other locations (50.0%, 42.9%, 33.3%, 30.8%, 21.4% and 20.0% for Java, Papua, Bali, Sulawesi, Timor, and Kalimantan, respectively, $P = 0.003$). Even after adjustment by age and sex in multivariate analysis, strains from Sumatera Island had significantly higher MNZ resistance rates than strains from other islands (OR = 7.9, OR = 10.5, OR = 15.3, OR = 28.3, OR = 17.5, and OR = 30.4 for Java, Papua, Bali, Sulawesi, Timor, and Kalimantan Island, respectively, $P < 0.05$).

Antibiotic resistance rate according to ethnic group

The predominant ethnic groups from different islands and their association with antibiotic resistance rates are shown in Table 4. Strains isolated from Ambonese, Chinese, and Balinese individuals displayed a high prevalence of CAM resistance (50.0%, 20.0%, and 16.6%, respectively, Table 4).

Only those from Buginese individuals showed AMX resistance >15%. Among three ethnicities with the highest prevalence of *H. pylori* in Indonesia [12], Papuan and Batak individuals were associated with a large number of antibiotic resistance types, including LVX, which is part of a second-line regimen, in contrast to strains isolated from Buginese individuals. Ambonese individuals were associated with higher rates of MNZ and TCN resistance than other ethnicities (both $P = 0.01$). Only strains from Dayak individuals were sensitive to all five antibiotics.

Multidrug resistance

No strain was resistant to all tested antibiotics (Table 5). Fifteen strains showed dual-drug resistance (11 strains for MNZ-LVX, two for CAM-LVX and one each for MNZ-AMX and LVX-AMX). Resistance to three antibiotics was observed in two (2.6%) strains that were isolated from Java Island; one was resistant to a combination of CAM, MNZ, and LVX.

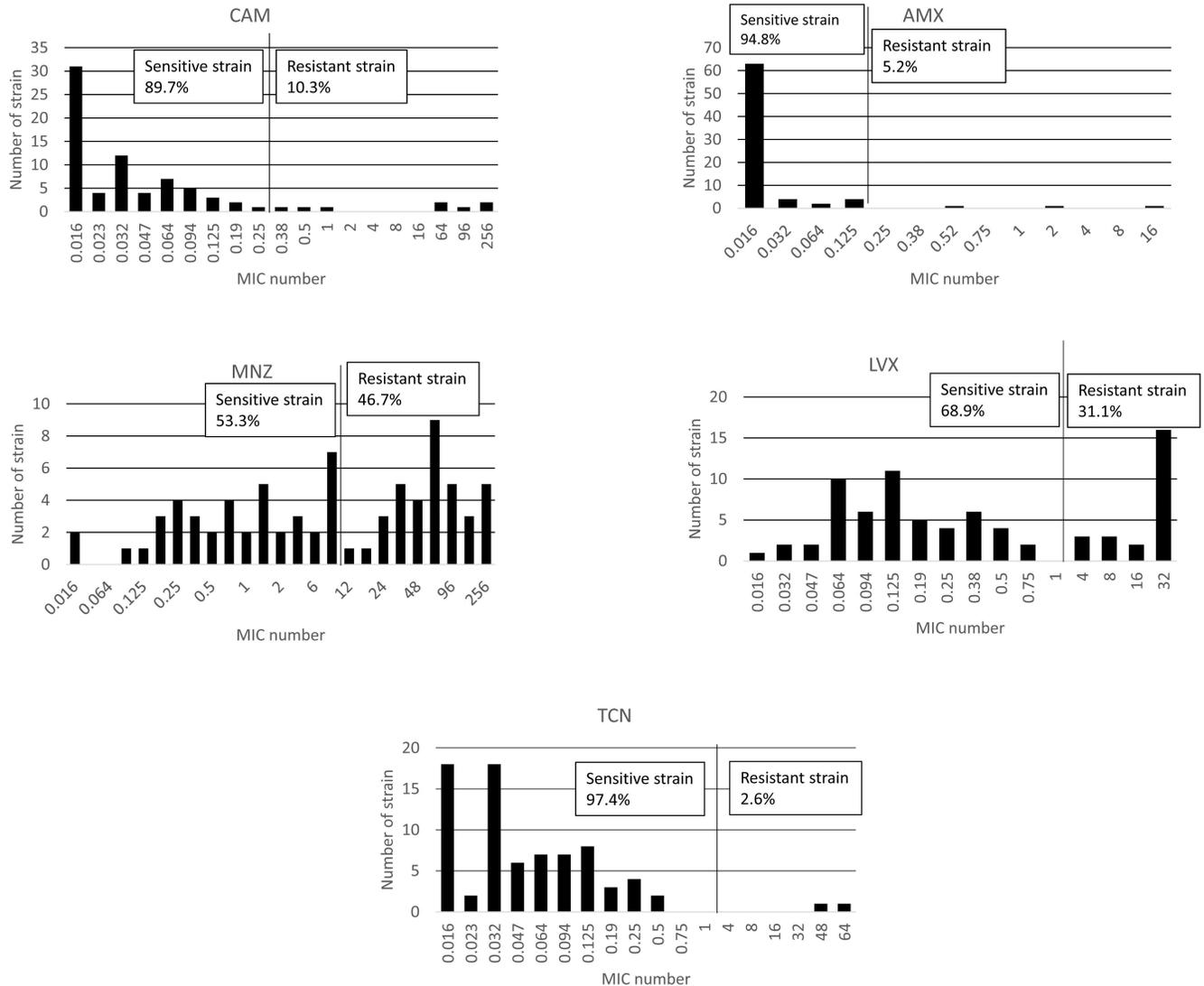


Fig 1. Distribution of antibiotic MIC values in Indonesia. The resistance rates to metronidazole and levofloxacin were high; in contrast, we revealed a low prevalence of clarithromycin, amoxicillin, and tetracycline resistance.

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In addition, two strains (obtained from Sumatera and Java Island) were recognized as resistant to four drugs including LVX. Overall, Java (six strains) and Sumatera Island (seven strains) showed a higher prevalence of multidrug resistance than other locations. No differences were observed for clinical outcomes between single-drug and multidrug resistant infections ($P = 0.53$).

Virulence genes of Indonesian strains and antibiotic resistance types

Table 6 shows the association between virulence genes and the pattern of antibiotic resistance. Although there was a significant association between TCN resistance and *cagA* positivity ($P = 0.004$), it was only 2 resistant strains which were insufficient to make conclusion. *jhp0562*-positive/ β -(1,3)*galT*-negative *H. pylori* types tended to have higher MNZ resistance than *jhp0562*-negative/ β -(1,3)*galT*-positive types ($P = 0.06$). There was no association between other virulence factors and antibiotics resistance types ($P > 0.05$).

Table 3. Prevalence of *H. pylori* antibiotic resistance in Indonesia by location.

| Island | N | Resistance (%) | | | | |
|------------|----|----------------|----------|-----------|----------|----------|
| | | CAM | AMX | MNZ* | LVX | TCN |
| Bali | 6 | 1 (16.7) | 0 (0.0) | 2 (33.3) | 1 (16.6) | 0 (0.0) |
| Java | 14 | 3 (21.4) | 0 (0.0) | 7 (50.0) | 7 (50.0) | 2 (14.3) |
| Kalimantan | 5 | 0 (0.0) | 0 (0.0) | 1 (20.0) | 1 (20.0) | 0 (0.0) |
| Papua | 7 | 1 (14.3) | 1 (14.3) | 3 (42.9) | 2 (28.6) | 0 (0.0) |
| Sulawesi | 13 | 1 (7.7) | 1 (7.7) | 4 (30.8) | 2 (15.4) | 0 (0.0) |
| Sumatera | 18 | 1 (5.6) | 1 (5.6) | 16 (88.9) | 8 (44.4) | 0 (0.0) |
| Timor | 14 | 0 (0.0) | 1 (7.1) | 3 (21.4) | 3 (21.4) | 0 (0.0) |

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

* Strains isolated from Sumatera Island had significantly higher MNZ resistance rates than strains isolated from other islands even after adjusting for age and sex ($P < 0.05$).

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Detection of *H. pylori* gene mutations and association with antimicrobial resistance

Two MNZ-resistant strains did not show identifiable specific bands for *rdxA* after targeted PCR; therefore, 34 MNZ-resistant and 5-sensitive control Indonesian strains were analyzed in this study. DNA sequence analysis of *rdxA* from all MNZ-sensitive strains revealed intact reading frames (lacking nonsense mutations). Pairwise alignment identified that the MNZ-sensitive strains shared 94.9–97.0% identity with the reference strain, 26695. In contrast, most MNZ-resistant strains contained missense mutations (16/34, 47.1%), resulting in amino acid substitutions (Table 7). In addition, five strains had nonsense mutations that resulted in the introduction of a premature stop codon. Moreover, the *rdxA* alleles of nine strains (26.5%) contained nucleotide deletions and/or insertions that resulted in a translational frameshift.

Table 4. Prevalence of antibiotic resistance in *H. pylori* in Indonesia by ethnicity.

| Ethnicity | Island | N | Resistance (%) | | | | |
|--------------|---------------------|----|----------------|----------|------------|----------|-----------|
| | | | CAM | AMX | MNZ | LVX | TCN |
| Javanese | Java | 3 | 0 (0.0) | 0 (0.0) | 1 (33.3) | 1 (33.3) | 0 (0.0) |
| Chinese** | Java and Kalimantan | 10 | 2 (20.0) | 0 (0.0) | 5 (50.0) | 5 (50.0) | 1 (10.0)* |
| Batak | Sumatera | 19 | 1 (5.2) | 1 (5.2) | 16 (84.2)* | 8 (42.1) | 0 (0.0) |
| Papuan | Papua | 7 | 1 (14.3) | 1 (14.3) | 3 (42.9) | 2 (28.6) | 0 (0.0) |
| Dayak | Kalimantan | 2 | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Buginese | Sulawesi | 6 | 0 (0.0) | 1 (16.6) | 2 (33.3) | 0 (0.0) | 0 (0.0) |
| Balinese | Bali | 6 | 1 (16.6) | 0 (0.0) | 2 (33.3) | 1 (16.6) | 0 (0.0) |
| Timor | Timor | 15 | 0 (0.0) | 1 (6.6) | 3 (20.0) | 4 (26.6) | 0 (0.0) |
| Minahasanese | Sulawesi | 7 | 1 (14.3) | 0 (0.0) | 2 (28.6) | 2 (28.6) | 0 (0.0) |
| Ambonese*** | Java | 2 | 1 (50.0) | 0 (0.0) | 2 (100.0)* | 1 (50.0) | 1 (50.0)* |

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

* $P < 0.05$

** Chinese-Indonesians are dispersing throughout the archipelago. In this study, the strains were obtained from Chinese individuals who lived in Surabaya, Java Island, Pontianak, and Kalimantan Island.

*** Ambonese are the predominant group of Ambon Island in Maluku, an island group east of Sulawesi. In this study, the strains were obtained from Ambonese who lived in Surabaya, Java Island.

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Table 5. Prevalence of multidrug resistance among Indonesian strains.

| Antibiotics | Total | Number of Patients (%) | | | | | | |
|-----------------------|-------|------------------------|------------------|-------------|-------------|-----------------|-----------------|--------------|
| | | Bali N = 6 | Kalimantan N = 5 | Java N = 14 | Papua N = 7 | Sulawesi N = 13 | Sumatera N = 18 | Timor N = 14 |
| Double drugs | | | | | | | | |
| MNZ + AMX | 1 | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (7.7) | 0 (0.0) | 0 (0.0) |
| MNZ + LVX | 11 | 1 (16.7) | 0 (0.0) | 2 (14.3) | 1 (14.3) | 0 (0.0) | 6 (33.3) | 1 (7.1) |
| CAM + LVX | 2 | 0 (0.0) | 0 (0.0) | 1 (7.1) | 0 (0.0) | 1 (7.7) | 0 (0.0) | 0 (0.0) |
| LVX + AMX | 1 | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (14.3) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Triple drugs | | | | | | | | |
| CAM + MNZ + LVX | 1 | 0 (0.0) | 0 (0.0) | 1 (7.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| TCN + MNZ + LVX | 1 | 0 (0.0) | 0 (0.0) | 1 (7.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Quadruple drugs | | | | | | | | |
| CAM + MNZ + AMX + LVX | 1 | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (5.6) | 0 (0.0) |
| CAM + MNZ + TCN + LVX | 1 | 0 (0.0) | 0 (0.0) | 1 (7.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |

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

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Among the five LVX-sensitive control Indonesian strains, no mutations in both *gyrA* and *gyrB* subunits were identified. In contrast, among 24 LVX-resistant strains, 22 had amino acid variants of the *gyrA* subunit (Table 8). Eleven LVX-resistant strains (45.8%) had an amino acid substitution at Asp-91, whereas six strains had an amino acid substitution at Asn-87. Both of these mutations (13/15, 86.7%) were predominantly associated with the highest MIC values observed (32 mg/L). In addition, two strains exhibited amino acid substitutions at Arg-484 and at Ser-479 in the *gyrB* subunit. When analyzing the association between these two genes and LVX-resistance, it was demonstrated the four strains (16.7%) had mutations in both *gyrA* and *gyrB*; 18/24 (75.0%) in *gyrA* only, and no strains had a mutation in *gyrB* only. We also found two of 24 (8.3%) LVX-resistant strains with no *gyrA* and *gyrB* mutations, which were associated with MIC values of 8 and 25 mg/L. There was no correlation between the degree of LVX resistance and the types or number of mutations in both genes.

Table 6. Association between virulence genes and antibiotic resistance pattern (%).

| Virulence Genes | CAM (%) | | MNZ (%) | | LVX (%) | |
|-------------------------|---------|-------|---------|------|---------|------|
| | S | R | S | R | S | R |
| Strain (number) | 70 | 7 | 41 | 36 | 53 | 24 |
| <i>cagA</i> positive | 94.3 | 100 | 95.1 | 94.4 | 96.2 | 91.7 |
| <i>cagA</i> type | | | | | | |
| • East-Asian-type | 60.0 | 85.7 | 53.7 | 72.2 | 58.5 | 70.8 |
| • Western-type | 25.7 | 0.0 | 29.3 | 16.7 | 28.3 | 12.5 |
| • ABB-type | 8.6 | 14.3 | 12.1 | 5.6 | 9.4 | 8.3 |
| <i>vacA</i> s1/m1 | 65.7 | 85.7 | 75.6 | 58.3 | 67.9 | 66.7 |
| <i>iceA1</i> genotype | 64.3 | 71.4 | 61.0 | 69.4 | 67.9 | 58.3 |
| <i>jhp0562</i> genotype | 20.0 | 0.0 | 24.4 | 11.1 | 22.6 | 8.3* |
| <i>oipA</i> "on" | 91.4 | 100.0 | 90.2 | 94.4 | 92.5 | 91.7 |
| <i>dupA</i> negative | 82.9 | 100 | 80.5 | 88.9 | 88.7 | 75.0 |

* P = 0.06 The data about the tetracycline and amoxicillin were excluded from the table, for which the resistant strains were 5 or less.

Abbreviations: AMX, amoxicillin; CAM, clarithromycin; MNZ, metronidazole; TCN, tetracycline; LVX, levofloxacin; *iceA1* genotype, *iceA1* positive/ *iceA2* negative; *jhp0562* genotype, *jhp0562* positive/ β -(1,3)*galT* negative.

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Table 7. Mutation type of *rdxA* related to metronidazole resistance.

| No | Strain | MIC | Sites of Mutation | Type of mutation |
|----|-------------|------|---|-----------------------|
| 1 | Jayapura16 | 32 | R16C, A183V | Missense mutation |
| 2 | Kupang23 | >256 | E15Q, R16H, E32D, E34stop | Premature stop codon |
| 3 | Kupang 30 | 96 | R90K, P106S, V111A | Missense mutation |
| 4 | Kupang 41 | 64 | Q6H, R16C, R90K, E175Q, N178D, A183V | Missense mutation |
| 5 | Kupang 5 | 12 | A68V | Missense mutation |
| 6 | Kupang 73 | 48 | K64N, H97T, P106S, H127Y, Q197stop | Premature stop codon |
| 7 | Malang1 | 48 | 16frameshift | Frameshift mutation |
| 8 | Manado26 | 64 | None | Non-specific mutation |
| 9 | Manado 31 | 48 | 65frameshift | Frameshift mutation |
| 10 | Medan3 | 16 | 65frameshift | Frameshift mutation |
| 11 | Medan10 | >256 | H97Y, P106T, G122S | Missense mutation |
| 12 | Medan15 | 64 | P51S, A68T, E175Q, R176H | Missense mutation |
| 13 | Medan17 | 128 | R16C, A68T, G122S, C159Y | Missense mutation |
| 14 | Medan18 | 64 | R90K, 195frameshift | Frameshift mutation |
| 15 | Medan19 | 128 | Q6H, R16C, R90K, H97T, A118S, E175Q, A183V, V204I | Missense mutation |
| 16 | Medan20 | 64 | K64N, H97Y, P106T, G122S, 137frameshift | Frameshift mutation |
| 17 | Medan22 | 32 | R16H, K64N, H97Y, P106M, G122S, E175Q | Missense mutation |
| 18 | Medan23 | 24 | V55M, K64N, G122S, E175Q, A183V, | Missense mutation |
| 19 | Medan25 | 64 | M1V, R16C, K64N, A80T, H97Y, P106T, G122S, E175Q | Missense mutation |
| 20 | Medan27 | >256 | 4frameshift | Frameshift mutation |
| 21 | Medan28 | 48 | P51S, K64N, M102stop, P106L, 173frameshift | Frameshift mutation |
| 22 | Medan30 | 96 | None | Non-specific mutation |
| 23 | Medan31 | 96 | K64N, H97I, P106T, G122S, E175stop | Premature stop codon |
| 24 | Medan32 | 32 | R10I, A68V, G145R | Missense mutation |
| 25 | Medan33 | 96 | R10S, H97Y, P106T, G122S, 169frameshift | Frameshift mutation |
| 26 | Makassar52 | 64 | None | Non-specific mutation |
| 27 | Makassar55 | 96 | Q6H, R16H, M56I, R90K, H97Y, P166S, | Missense mutation |
| 28 | Pontianak20 | >256 | G163D, E173stop | Premature stop codon |
| 29 | Surabaya68 | 24 | R16H, P106T, V204I | Missense mutation |
| 30 | Surabaya69 | 32 | None | Non-specific mutation |
| 31 | Surabaya79 | 128 | Q6H, R16C, M56I, A80T, R90K, H97Y | Missense mutation |
| 32 | Surabaya137 | >256 | 23frameshift | Frameshift mutation |
| 33 | Surabaya283 | 32 | Q146stop | Premature stop codon |
| 34 | Surabaya304 | 64 | I182V | Missense mutation |

R16C means Cysteine replaced Arginine amino acid in the position 16; E34stop means stop codon replaced Glutamate amino acid in the position 34; 16frameshift means frameshift mutation in the position 16.

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Based on 23Sr RNA sequencing in the seven CAM-resistant strains, three (42.9%) exhibited an interesting point mutation, specifically A2143G (Table 9), and two of these strains were associated with high MIC values (64 and 96 mg/L). In contrast, we found minimal nucleotide variation on the one control Indonesian CAM-sensitive strain. Interestingly, there was no specific mutation in the strain associated with the highest MIC values (Surabaya137). Based on the previous report [21], we performed next generation sequencing of the Surabaya137 strain (average sequencing depth was 46.9x and overall %GC was 38.4). Using strain 26695 and the control CAM-sensitive strain Medan27, we could not identify any mutations in full-length 23S

Table 8. Mutation type of *gyrA* and *gyrB* related to levofloxacin resistance.

| No | Strain | MIC (mg/L) | <i>gyrA</i> mutation | <i>gyrB</i> mutation |
|----|-------------|------------|----------------------|----------------------|
| 1 | Jayapura1 | >32 | N87K | None |
| 2 | Jayapura21 | >32 | N87K | None |
| 3 | Kupang2 | 4 | D91N, A129T | S479G |
| 4 | Kupang11 | >32 | D91Y | None |
| 5 | Kupang23 | >32 | A129T | S479G |
| 6 | Kupang41 | 8 | D91N | R484K |
| 7 | Malang1 | 16 | D91N | None |
| 8 | Manado18 | 8 | None | None |
| 9 | Manado20 | 8 | D91Y | None |
| 10 | Medan3 | >32 | N87I | None |
| 11 | Medan 10 | 25 | None | None |
| 12 | Medan15 | >32 | R140K, D192N | None |
| 13 | Medan 17 | 16 | D34N | None |
| 14 | Medan18 | 4 | D91G, D161N | None |
| 15 | Medan22 | >32 | D91N | None |
| 16 | Medan 23 | 4 | D34Y, R140K | None |
| 17 | Medan30 | >32 | D91N | None |
| 18 | Pontianak50 | >32 | D91G | None |
| 19 | Surabaya71 | >32 | D91N | None |
| 20 | Surabaya79 | >32 | N87Y | R484K |
| 21 | Surabaya137 | >32 | N87K | None |
| 22 | Surabaya151 | >32 | N87K | None |
| 23 | Surabaya283 | >32 | D91Y | None |
| 24 | Surabaya304 | >32 | D91G | None |

N87K means Lysine replaced Asparagine amino acid in the position 87.

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rRNA. Although we found point mutations, (T189C and T198C) in *hp1048* (*infB*), these were silent mutations. In contrast, compared to strains 29965 and Medan27, we confirmed the involvement of novel mutated sequences in *hp1314* (*rpl22*) including 19 bp deletions at position 535 (Table 9).

Table 9. Gene mutations related to clarithromycin resistance.

| No | Strain | MIC (mg/L) | <i>23SrRNA</i> | <i>rpl22</i> (<i>hp1314</i>) | <i>infB</i> (<i>hp1048</i>) |
|----|-------------|------------|----------------|--------------------------------|--|
| 1 | Jayapura6 | 96 | A2143G | - | - |
| 2 | Kupang64 | 32 | None | - | - |
| 3 | Manado20 | 64 | None | - | - |
| 4 | Medan15 | 0.5 | None | - | - |
| 5 | Surabaya71 | 64 | A2143G | - | - |
| 6 | Surabaya137 | >256 | None | None | A352G, C361T, G374A, A391G, C406A, T437C, G529A, C634D, G635T, G671A, T672C, A673T, 535del, T946C, G995T, G2245A |
| 7 | Surabaya304 | 1 | A2143G, G2172T | - | - |

A2143G means Guanine replaced Adenosine in the position 2143.

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Nucleotide sequencing

Nucleotide sequence data reported are available under the DDBJ accession numbers LC174777-LC174814 and LC175851 (*rdxA*), LC174815-LC174843 (*gyrB*), LC174844-LC174852 and LC174855-LC174874 (*gyrA*), LC175228-LC175232 and LC175234-LC175236 (*23srRNA*), LC175237-LC175238 (*infB*), LC175239-LC175240 (*rpl22*), LC174875-LC174908 (*cagA*) and LC175814-LC175850 (*oipA*).

Discussion

Southeast Asia is a region with low CAM resistance rates [2]. The previous studies reported that CAM resistance rates among *H. pylori* isolates of Thailand and Malaysia, neighboring countries, were very low (3.7% and 5.2%, respectively) [18,39]. In agreement with this data, we revealed a low prevalence of CAM resistance. It is suggested that CAM-based triple therapy might still be useful as an initial treatment of *H. pylori* infection in Indonesia. However, further analysis based on the ethnicity of the host revealed that Ambonese, Chinese, and Balinese individuals were associated with strains with CAM resistance rates, exceeding those of the recommendations from the Maastricht guidelines (>15–20%) [37,38]. Furthermore, breakdown based on location showed that strains isolated from Bali and Java Island demonstrated high CAM resistance. Our report was consistent with a previous study in Indonesia, in which isolates from Java Island had a CAM resistance rate of 27.8% [15]. A recent report from Singapore, a neighboring country, also demonstrated a changing CAM-resistance profile over 15 years (7.9–17.1%) [40]. Therefore, triple therapy with CAM should be utilized with caution or should be culture-based in some areas, and involving particular ethnicities, in Indonesia.

We identified a mononucleotide substitution from A to G at site 2143 in CAM-resistant strains. Moreover, this was associated with high CAM MIC values (strains Jayapura6 and Surabaya304). A previous study reported the A2143G mutation has a much stronger impact on CAM resistance than the A2142G and A2142C mutations [41]. However, we failed to identify T2183C and A2223G mutations, which are frequently found to be the cause of CAM resistance in Asian, rather than in Western, countries [19]. Our current study also confirmed that *hp1314* (*rpl22*) mutations are associated with CAM resistance [21], although we identified different mutation types compared to those of Vietnamese strains (3 bp deletion and 9 bp insertion in *rpl22*). In contrast to the current study, we previously demonstrated that a single mutation in *infB* or *rpl22* resulted in low MIC values and showed the involvement of *infB*, *rpl22*, and *23S rRNA* in higher MIC values. Therefore, we suggested that *rpl22* mutations might not only result in synergistic effects, but also could be independent causes of CAM resistance.

Aside from isolates from Buginese individuals, for which the AMX resistance rate was greater than 15%, in general, our study revealed that Indonesian isolates had low resistance to AMX. We found only one resistant strain each from four islands. This is in contrast to the previous report in Indonesia [15], but similar to other typical Southeast Asian antibiotic resistance patterns [2]. This distinction might be based on differences in research facility reproducibility, caused by a lack of standardized testing protocols or different regional practices. Thus, AMX might be useful as a secondary antibiotic, for use in cases exhibiting poor responses to CAM-based triple therapy in Indonesia. This drug has been the first antibiotic utilized for *H. pylori* treatment because of the assumed absence of resistance [42]. Nevertheless, increasing primary AMX-resistance rates have been reported in South Korea (7.1–18.5%) [43], and in India and Pakistan (72.5% and 37.0%, respectively) [44,45]. Additionally, AMX can be obtained without prescription and has been one of the most commonly used antibiotics in Indonesia in recent years. A strict policy for antibiotic use is necessary to counteract the failure of primary antibiotic treatment for *H. pylori* in Indonesia.

TCN is an anti-microbial, to which resistance is occasionally experienced. The reason for this is that to achieve resistance, three point changes are required [9]. In agreement with most countries [46], we detected low TCN resistance in Indonesian strains. Although Ambonese individuals were associated with significantly higher resistance than strains isolated from other locations, the observation of only two resistant strains is of some concern. This is the first study to examine the TCN resistance rate in Indonesia. Due to the important role of TCN as a salvage quadruple therapy, our data are vital for guiding second line regimens for eliminating *H. pylori* infections.

The resistance rate for MNZ was high in Indonesia. Moreover, two *H. pylori* groups, specifically, Batak and Papua, had rates higher than the preferential number outlined by the Maas-tricht III Consensus Report (>40%) [37]. However, in Papua, the number of strains was small. In Asia, only Japan, Thailand, and Malaysia have populations associated with <40% MNZ resistance [2]. Therefore, regimens including MNZ are not suitable and should not be chosen as first-line treatments in Indonesia. We distinguished diverse mutations involving *rdxA* in most MNZ-resistant strains. Complex genetic events (insertions, deletions, and missense and frameshift mutations) were simultaneously present in the strains. Previously it was shown that only 11.8% of MNZ-resistant strains did not harbor any mutation in *rdxA*, and this was probably related to *frxA* [22], *rpsU* [47], *dppA* or *dapF* [48] alterations. Conversely, MNZ-sensitive strains had high resemblance against reference strains. Because of different mutations in *rdxA*, molecular antibiotic susceptibility testing is not applicable for metronidazole.

LVX was proposed a decade ago based on its role in salvage treatment regimens after the failure of clarithromycin-based treatments [49]. Nevertheless, the frequency of LVX resistance is by all accounts expanding around the world, which might diminish the efficacy LVX-based treatment regimens [50,51]. Our findings showed a high prevalence of primary resistance to LVX (33.1%). Similarly, LVX is not sufficiently effective for inclusion in treatment regimens in Indonesia. Point mutations in the quinolones resistance-determining region (QRDR) of *gyrA* abrogate binding between the antibiotic and the enzyme, resulting in bacterial antibiotic resistance [52]. In the present study we found the predominant mutations at amino acid 87 (Asn to Lys, Tyr, or Ile) and 91 (Asp to Asn, Gly, or Tyr), which have previously been described [18,53,54]. Additionally, these point mutations were present at a high frequency (86.7%) and were associated with high MIC values. Although we found amino acid substitutions at Arg-484 and at Ser-479 in *gyrB* subunits, they had were associated with the *gyrA* mutations in amino acid 87/91, which most likely minimizes the influence of these *gyrB* mutations in Indonesian LVX-resistant strains. Therefore, in Indonesia, screening for *gyrA* mutations could be adequate for recognizing LVX-resistant strains.

The number of *H. pylori* strains demonstrating triple or quadruple resistance in this study appeared to be a serious challenge in the fight against infections and a hindrance to the success of eradication regimens. Java and Sumatera Island could be two locations with a higher risk for *H. pylori* treatment failure in Indonesia due to the associated high antibiotic resistance type area. Several regions of Indonesia are associated with a high prevalence of *H. pylori* infections; increased resistance to the antibiotics used to treat this bacterium might result in increased recurrence rates. It is therefore important to perform susceptibility-guided re-treatment using a case-by-case approach, if available, in patients demonstrating initial treatment failure. Recently, a high accuracy DNA strip genotyping test was developed combining PCR and hybridization that permits the molecular identification of mutations in *gyrA* and 23S *rRNA* within 6 h [55]. Our genotypic resistance results are vital to guide follow-up treatment protocols after first-line regimens fail.

The number of samples in this study was relatively low, which certainly suggests the major limitations of this study. We considered that a larger sample size among region is necessary to

elucidate the prevalence of *H. pylori* antibiotic resistance in Indonesia. The current study is a pilot study for a larger survey and we are now continuing the similar surveys to that performed in this study, to increase sample numbers and expand geographically to other islands. In addition, we only determined the presence of well-known genetic mutations associated with antibiotic resistance. *H. pylori* contains approximately 1,600 genes, and it is likely that only a fraction of genomic changes that are related to drug resistance have been identified. Next-generation sequencing technology is beneficial in that it can yield enormous numbers of DNA sequences in less time and at lower cost, which could be used to clarify the evolution and pathogenicity of *H. pylori*. To guide antibiotic regimens in Indonesia, the locations were perhaps more important than the ethnicities of the patients. Most antibiotic resistance is related to local antibiotic consumption [56]. Moreover, such resistance is primarily due to the *H. pylori* genotype, rather than the human genotype.

Conclusions

The rates of resistance to MNZ and LVX were high in Indonesia, which implies that MNZ-, and LVX-based triple therapies are not valuable for first-line treatment of *H. pylori* in Indonesia. In general, we revealed a low prevalence of CAM, AMX, and TCN resistance. Nevertheless, individuals of several ethnicities were shown to be associated with a high prevalence of CAM, TC, and MNZ resistance. In this manner, CAM- or MNZ-based triple therapy should be used with caution or should be demographic-based in some regions of Indonesia. National epidemiological surveillance of resistance rates is required to further determine optimal treatment strategies in Indonesia.

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References

1. Peek RM, Blaser MJ. *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. *Nat Rev Cancer*. 2002; 2: 28–37. doi: [10.1038/nrc703](https://doi.org/10.1038/nrc703) PMID: [11902583](https://pubmed.ncbi.nlm.nih.gov/11902583/)
2. Miftahussurur M, Yamaoka Y. Appropriate first-line regimens to combat *Helicobacter pylori* antibiotic resistance: an Asian perspective. *Molecules*. 2015; 20: 6068–6092. doi: [10.3390/molecules20046068](https://doi.org/10.3390/molecules20046068) PMID: [25856059](https://pubmed.ncbi.nlm.nih.gov/25856059/)
3. Miwa H, Go MF, Sato N. *H. pylori* and gastric cancer: the Asian enigma. *Am J Gastroenterol*. 2002; 97: 1106–1112. doi: [10.1111/j.1572-0241.2002.05663.x](https://doi.org/10.1111/j.1572-0241.2002.05663.x) PMID: [12014714](https://pubmed.ncbi.nlm.nih.gov/12014714/)
4. Fock KM, Ang TL. Epidemiology of *Helicobacter pylori* infection and gastric cancer in Asia. *J Gastroenterol Hepatol*. 2010; 25: 479–486. doi: [10.1111/j.1440-1746.2009.06188.x](https://doi.org/10.1111/j.1440-1746.2009.06188.x) PMID: [20370726](https://pubmed.ncbi.nlm.nih.gov/20370726/)
5. Fock KM, Katelaris P, Sugano K, Ang TL, Hunt R, Talley NJ, et al. Second Asia-Pacific Consensus Guidelines for *Helicobacter pylori* infection. *J Gastroenterol Hepatol*. 2009; 24: 1587–1600. doi: [10.1111/j.1440-1746.2009.05982.x](https://doi.org/10.1111/j.1440-1746.2009.05982.x) PMID: [19788600](https://pubmed.ncbi.nlm.nih.gov/19788600/)
6. Kim SG, Jung HK, Lee HL, Jang JY, Lee H, Kim CG, et al. [Guidelines for the diagnosis and treatment of *Helicobacter pylori* infection in Korea, 2013 revised edition]. *Korean J Gastroenterol*. 2013; 62: 3–26. PMID: [23954956](https://pubmed.ncbi.nlm.nih.gov/23954956/)
7. Asaka M. A new approach for elimination of gastric cancer deaths in Japan. *Int J Cancer*. 2013; 132: 1272–1276. doi: [10.1002/ijc.27965](https://doi.org/10.1002/ijc.27965) PMID: [23180638](https://pubmed.ncbi.nlm.nih.gov/23180638/)
8. Chinese Society of Gastroenterology CSGoHp, Liu WZ, Xie Y, Cheng H, Lu NH, Hu FL, et al. Fourth Chinese National Consensus Report on the management of *Helicobacter pylori* infection. *J Dig Dis*. 2013; 14: 211–221. doi: [10.1111/1751-2980.12034](https://doi.org/10.1111/1751-2980.12034) PMID: [23302262](https://pubmed.ncbi.nlm.nih.gov/23302262/)
9. Megraud F. The challenge of *Helicobacter pylori* resistance to antibiotics: the comeback of bismuth-based quadruple therapy. *Therap Adv Gastroenterol*. 2012; 5: 103–109. doi: [10.1177/1756283X11432492](https://doi.org/10.1177/1756283X11432492) PMID: [22423259](https://pubmed.ncbi.nlm.nih.gov/22423259/)
10. Abdullah AA, Abdullah M, Fauzi A, Syam AF, Simadibrata M, Makmun D. The effectiveness of endoscopic retrograde cholangiopancreatography in the management of patients with jaundice at Cipto Mangunkusumo Hospital, Jakarta. *Acta Med Indones*. 2012; 44: 298–303. PMID: [23314970](https://pubmed.ncbi.nlm.nih.gov/23314970/)
11. Miftahussurur M, Shiota S, Suzuki R, Matsuda M, Uchida T, Kido Y, et al. Identification of *Helicobacter pylori* infection in symptomatic patients in Surabaya, Indonesia, using five diagnostic tests. *Epidemiol Infect*. 2015; 143: 986–996. doi: [10.1017/S095026881400154X](https://doi.org/10.1017/S095026881400154X) PMID: [25034254](https://pubmed.ncbi.nlm.nih.gov/25034254/)
12. Syam AF, Miftahussurur M, Makmun D, Nusi IA, Zain LH, Zulkhairi, et al. Risk Factors and Prevalence of *Helicobacter pylori* in Five Largest Islands of Indonesia: A Preliminary Study. *PLoS One*. 2015; 10: e0140186. doi: [10.1371/journal.pone.0140186](https://doi.org/10.1371/journal.pone.0140186) PMID: [26599790](https://pubmed.ncbi.nlm.nih.gov/26599790/)
13. Miftahussurur M, Syam AF, Makmun D, Nusi IA, Zein LH, Zulkhairi, et al. *Helicobacter pylori* virulence genes in the five largest islands of Indonesia. *Gut Pathog*. 2015; 7: 26. doi: [10.1186/s13099-015-0072-2](https://doi.org/10.1186/s13099-015-0072-2) PMID: [26442711](https://pubmed.ncbi.nlm.nih.gov/26442711/)
14. Sugano K, Tack J, Kuipers EJ, Graham DY, El-Omar EM, Miura S, et al. Kyoto global consensus report on *Helicobacter pylori* gastritis. *Gut*. 2015; 64: 1353–1367. doi: [10.1136/gutjnl-2015-309252](https://doi.org/10.1136/gutjnl-2015-309252) PMID: [26187502](https://pubmed.ncbi.nlm.nih.gov/26187502/)
15. Kumala W, Rani A. Patterns of *Helicobacter pylori* isolate resistance to fluoroquinolones, amoxicillin, clarithromycin and metronidazoles. *Southeast Asian J Trop Med Public Health*. 2006; 37: 970–974. PMID: [17333742](https://pubmed.ncbi.nlm.nih.gov/17333742/)
16. Kobayashi I, Murakami K, Kato M, Kato S, Azuma T, Takahashi S, et al. Changing antimicrobial susceptibility epidemiology of *Helicobacter pylori* strains in Japan between 2002 and 2005. *J Clin Microbiol*. 2007; 45: 4006–4010. doi: [10.1128/JCM.00740-07](https://doi.org/10.1128/JCM.00740-07) PMID: [17942652](https://pubmed.ncbi.nlm.nih.gov/17942652/)
17. Cuadrado-Lavin A, Salcines-Caviedes JR, Carrascosa MF, Mellado P, Monteagudo I, Llorca J, et al. Antimicrobial susceptibility of *Helicobacter pylori* to six antibiotics currently used in Spain. *J Antimicrob Chemother*. 2012; 67: 170–173. PMID: [21965436](https://pubmed.ncbi.nlm.nih.gov/21965436/)
18. Teh X, Khosravi Y, Lee WC, Leow AH, Loke MF, Vadivelu J, et al. Functional and molecular surveillance of *Helicobacter pylori* antibiotic resistance in Kuala Lumpur. *PLoS One*. 2014; 9: e101481. doi: [10.1371/journal.pone.0101481](https://doi.org/10.1371/journal.pone.0101481) PMID: [25003707](https://pubmed.ncbi.nlm.nih.gov/25003707/)
19. Ierardi E, Giorgio F, Losurdo G, Di Leo A, Principi M. How antibiotic resistances could change *Helicobacter pylori* treatment: A matter of geography? *World J Gastroenterol*. 2013; 19: 8168–8180. doi: [10.3748/wjg.v19.i45.8168](https://doi.org/10.3748/wjg.v19.i45.8168) PMID: [24363506](https://pubmed.ncbi.nlm.nih.gov/24363506/)
20. Oleastro M, Ménard A, Santos A, Lamouliatte H, Monteiro L, Barthélémy P, et al. Real-time PCR assay for rapid and accurate detection of point mutations conferring resistance to clarithromycin in *Helicobacter pylori*. *J Clin Microbiol*. 2003; 41: 397–402. doi: [10.1128/JCM.41.1.397-402.2003](https://doi.org/10.1128/JCM.41.1.397-402.2003) PMID: [12517879](https://pubmed.ncbi.nlm.nih.gov/12517879/)

21. Binh TT, Shiota S, Suzuki R, Matsuda M, Trang TT, Kwon DH, et al. Discovery of novel mutations for clarithromycin resistance in *Helicobacter pylori* by using next-generation sequencing. *J Antimicrob Chemother.* 2014; 69: 1796–1803. PMID: [24648504](#)
22. Gerrits MM, van der Wouden EJ, Bax DA, van Zwet AA, van Vliet AH, de Jong A, et al. Role of the *rdxA* and *frxA* genes in oxygen-dependent metronidazole resistance of *Helicobacter pylori*. *J Med Microbiol.* 2004; 53: 1123–1128. doi: [10.1099/jmm.0.45701-0](#) PMID: [15496391](#)
23. Rimbara E, Noguchi N, Kawai T, Sasatsu M. Fluoroquinolone resistance in *Helicobacter pylori*: role of mutations at position 87 and 91 of GyrA on the level of resistance and identification of a resistance conferring mutation in GyrB. *Helicobacter.* 2012; 17: 36–42.
24. Kwon DH, Pena JA, Osato MS, Fox JG, Graham DY, Versalovic J. Frameshift mutations in *rdxA* and metronidazole resistance in North American *Helicobacter pylori* isolates. *J Antimicrob Chemother.* 2000; 46: 793–796. PMID: [11062200](#)
25. Wang LH, Cheng H, Hu FL, Li J. Distribution of *gyrA* mutations in fluoroquinolone-resistant *Helicobacter pylori* strains. *World J Gastroenterol.* 2010; 16: 2272–2277. doi: [10.3748/wjg.v16.i18.2272](#) PMID: [20458765](#)
26. Ho SL, Tan EL, Sam CK, Goh KL. Clarithromycin resistance and point mutations in the *23S rRNA* gene in *Helicobacter pylori* isolates from Malaysia. *J Dig Dis.* 2010; 11: 101–105. doi: [10.1111/j.1751-2980.2010.00423.x](#) PMID: [20402836](#)
27. Matsunari O, Shiota S, Suzuki R, Watada M, Kinjo N, Murakami K, et al. Association between *Helicobacter pylori* virulence factors and gastroduodenal diseases in Okinawa, Japan. *J Clin Microbiol.* 2012; 50: 876–883. doi: [10.1128/JCM.05562-11](#) PMID: [22189111](#)
28. Uchida T, Nguyen LT, Takayama A, Okimoto T, Kodama M, Murakami K, et al. Analysis of virulence factors of *Helicobacter pylori* isolated from a Vietnamese population. *BMC Microbiol.* 2009; 9: 175. doi: [10.1186/1471-2180-9-175](#) PMID: [19698173](#)
29. Yamaoka Y, Kikuchi S, el-Zimaity H, Gutierrez O, Osato M, Graham D. Importance of *Helicobacter pylori oipA* in clinical presentation, gastric inflammation, and mucosal interleukin 8 production. *Gastroenterology.* 2002; 123: 414–424. PMID: [12145793](#)
30. Oleastro M, Monteiro L, Lehours P, Mégraud F, Ménard A. Identification of markers for *Helicobacter pylori* strains isolated from children with peptic ulcer disease by suppressive subtractive hybridization. *Infect Immun.* 2006; 74: 4064–4074. doi: [10.1128/IAI.00123-06](#) PMID: [16790780](#)
31. Atherton JC, Cao P, Peek RM, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific *vacA* types with cytotoxin production and peptic ulceration. *J Biol Chem.* 1995; 270: 17771–17777. PMID: [7629077](#)
32. Yamaoka Y, El-Zimaity H, Gutierrez O, Figura N, Kim J, Kodama T, et al. Relationship between the *cagA* 3' repeat region of *Helicobacter pylori*, gastric histology, and susceptibility to low pH. *Gastroenterology.* 1999; 117: 342–349. PMID: [10419915](#)
33. Yamaoka Y, Kodama T, Gutierrez O, Kim JG, Kashima K, Graham DY. Relationship between *Helicobacter pylori iceA*, *cagA*, and *vacA* status and clinical outcome: studies in four different countries. *J Clin Microbiol.* 1999; 37: 2274–2279. PMID: [10364597](#)
34. Takahashi A, Shiota S, Matsunari O, Watada M, Suzuki R, Nakachi S, et al. Intact Long-Type *dupA* as a Marker for Gastroduodenal Diseases in Okinawan Subpopulation, Japan. *Helicobacter.* 2012.
35. Rhead JL, Letley DP, Mohammadi M, Hussein N, Mohagheghi MA, Eshagh Hosseini M, et al. A new *Helicobacter pylori* vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. *Gastroenterology.* 2007; 133: 926–936. doi: [10.1053/j.gastro.2007.06.056](#) PMID: [17854597](#)
36. De Francesco V, Giorgio F, Hassan C, Manes G, Vannella L, Panella C, et al. Worldwide *H. pylori* antibiotic resistance: a systematic review. *J Gastrointest Liver Dis.* 2010; 19: 409–414. PMID: [21188333](#)
37. Malfertheiner P, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, et al. Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III Consensus Report. *Gut.* 2007; 56: 772–781. doi: [10.1136/gut.2006.101634](#) PMID: [17170018](#)
38. Malfertheiner P, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, et al. Management of *Helicobacter pylori* infection—the Maastricht IV/ Florence Consensus Report. *Gut.* 2012; 61: 646–664. doi: [10.1136/gutjnl-2012-302084](#) PMID: [22491499](#)
39. Vilaichone RK, Gumnarai P, Ratanachu-Ek T, Mahachai V. Nationwide survey of *Helicobacter pylori* antibiotic resistance in Thailand. *Diagn Microbiol Infect Dis.* 2013; 77: 346–349. doi: [10.1016/j.diagmicrobio.2013.08.010](#) PMID: [24094837](#)
40. Ang TL, Fock KM, Ang D, Kwek AB, Teo EK, Dhamodaran S. The Changing Profile of *Helicobacter pylori* Antibiotic Resistance in Singapore: A 15-Year Study. *Helicobacter.* 2016.

41. De Francesco V, Margiotta M, Zullo A, Hassan C, Troiani L, Burattini O, et al. Clarithromycin-resistant genotypes and eradication of *Helicobacter pylori*. *Ann Intern Med*. 2006; 144: 94–100. PMID: [16418408](#)
42. Francesco VD, Zullo A, Hassan C, Giorgio F, Rosania R, Ierardi E. Mechanisms of *Helicobacter pylori* antibiotic resistance: An updated appraisal. *World J Gastrointest Pathophysiol*. 2011; 2: 35–41. doi: [10.4291/wjgp.v2.i3.35](#) PMID: [21860834](#)
43. Lee JW, Kim N, Kim JM, Nam RH, Chang H, Kim JY, et al. Prevalence of primary and secondary antimicrobial resistance of *Helicobacter pylori* in Korea from 2003 through 2012. *Helicobacter*. 2013; 18: 206–214. doi: [10.1111/hel.12031](#) PMID: [23241101](#)
44. Pandya HB, Agravat HH, Patel JS, Sodagar N. Emerging antimicrobial resistance pattern of *Helicobacter pylori* in central Gujarat. *Indian J Med Microbiol*. 2014; 32: 408–413. doi: [10.4103/0255-0857.142256](#) PMID: [25297026](#)
45. Khan A, Farooqui A, Manzoor H, Akhtar SS, Quraishy MS, Kazmi SU. Antibiotic resistance and *cagA* gene correlation: a looming crisis of *Helicobacter pylori*. *World J Gastroenterol*. 2012; 18: 2245–2252. doi: [10.3748/wjg.v18.i18.2245](#) PMID: [22611319](#)
46. Megraud F. Epidemiology and mechanism of antibiotic resistance in *Helicobacter pylori*. *Gastroenterology*. 1998; 115: 1278–1282. PMID: [9797385](#)
47. Binh TT, Suzuki R, Trang TT, Kwon DH, Yamaoka Y. Search for Novel Candidate Mutations for Metronidazole Resistance in *Helicobacter pylori* Using Next-Generation Sequencing. *Antimicrob Agents Chemother*. 2015.
48. Li AQ, Dai N, Yan J, Zhu YL. Screening for metronidazole-resistance associated gene fragments of *H pylori* by suppression subtractive hybridization. *World J Gastroenterol*. 2007; 13: 1847–1850. PMID: [17465479](#)
49. Cammarota G, Cianci R, Cannizzaro O, Cuoco L, Pirozzi G, Gasbarrini A, et al. Efficacy of two one-week rabeprazole/levofloxacin-based triple therapies for *Helicobacter pylori* infection. *Aliment Pharmacol Ther*. 2000; 14: 1339–1343. PMID: [11012480](#)
50. Glocker E, Stueger HP, Kist M. Quinolone resistance in *Helicobacter pylori* isolates in Germany. *Antimicrob Agents Chemother*. 2007; 51: 346–349. doi: [10.1128/AAC.00614-06](#) PMID: [17043117](#)
51. Matsumoto Y, Miki I, Aoyama N, Shirasaka D, Watanabe Y, Morita Y, et al. Levofloxacin- versus metronidazole-based rescue therapy for *H. pylori* infection in Japan. *Dig Liver Dis*. 2005; 37: 821–825. doi: [10.1016/j.dld.2005.06.002](#) PMID: [16040284](#)
52. Nishizawa T, Suzuki H, Hibi T. Quinolone-Based Third-Line Therapy for *Helicobacter pylori* Eradication. *J Clin Biochem Nutr*. 2009; 44: 119–124. doi: [10.3164/jcbn.08-220R](#) PMID: [19308265](#)
53. Nishizawa T, Suzuki H, Kurabayashi K, Masaoka T, Muraoka H, Mori M, et al. Gatifloxacin resistance and mutations in *gyra* after unsuccessful *Helicobacter pylori* eradication in Japan. *Antimicrob Agents Chemother*. 2006; 50: 1538–1540. doi: [10.1128/AAC.50.4.1538-1540.2006](#) PMID: [16569878](#)
54. Garcia M, Raymond J, Garnier M, Cremniter J, Burucoa C. Distribution of spontaneous *gyrA* mutations in 97 fluoroquinolone-resistant *Helicobacter pylori* isolates collected in France. *Antimicrob Agents Chemother*. 2012; 56: 550–551. doi: [10.1128/AAC.05243-11](#) PMID: [22064536](#)
55. Cambau E, Allerheiligen V, Coulon C, Corbel C, Lascols C, Deforges L, et al. Evaluation of a new test, genotype HelicoDR, for molecular detection of antibiotic resistance in *Helicobacter pylori*. *J Clin Microbiol*. 2009; 47: 3600–3607. doi: [10.1128/JCM.00744-09](#) PMID: [19759218](#)
56. Megraud F, Coenen S, Versporten A, Kist M, Lopez-Brea M, Hirschl AM, et al. *Helicobacter pylori* resistance to antibiotics in Europe and its relationship to antibiotic consumption. *Gut*. 2013; 62: 34–42. doi: [10.1136/gutjnl-2012-302254](#) PMID: [22580412](#)