The triple therapy used to treat patients with nonulcer dyspepsia (NUD) also harbor the bacterium. It was noted that a substantial number of subjects that is closely associated with gastritis and peptic ulcer (PU) diseases. The isolates from the 120 specimens were tested by the agar dilution method, and 38 (31.7%) were found to be metronidazole resistant. Among the 211 subjects, 81 of 115 (70.4%) patients with peptic ulcer (PU) were infected with *H. pylori*, whereas 39 of 96 (40.6%) patients with nonulcer dyspepsia (NUD) were infected with *H. pylori*. Interestingly, a significantly more NUD patients than PU patients harbored metronidazole-resistant *H. pylori* (22 of 39 [56.4%] and 16 of 81 [19.8%], respectively; \( P < 0.001 \)). A similar pattern was also observed among NUD patients of different ethnicities but not between male and female patients (23 of 78 [29.5%] and 15 of 42 [35.7%], respectively; \( P = 0.54 \)). In the posttreatment follow-up, five of six patients who had positive urea breath test results, indicating treatment failure, were NUD patients. Of these, four harbored metronidazole-resistant *H. pylori* strains. This further illustrates the relevance of metronidazole-resistant *H. pylori* in NUD patients. The significantly higher percentage of metronidazole-resistant *H. pylori* isolates in NUD patients may be attributed to the protection offered by the mucus layer of the nonulcerated stomach to the bacteria that reside below it, resulting in organism exposure to sublethal concentrations of metronidazole and leading to the induction of metronidazole resistance. The results demonstrate that the *H. pylori* isolates colonizing NUD patients are more likely to be resistant to metronidazole. It will therefore be useful to reevaluate the use of metronidazole in the treatment of NUD patients infected with *H. pylori*.

### Helicobacter pylori

*Helicobacter pylori* is a gram-negative spiral microorganism that is closely associated with gastritis and peptic ulcer (PU) diseases. It was noted that a substantial number of subjects with nonulcer dyspepsia (NUD) also harbor the bacterium. The triple therapy used to treat *H. pylori* infection, consisting of bismuth subsalicylate and two antibiotics, usually metronidazole and tetracycline or amoxicillin, has been found to result in an eradication rate of approximately 90% (18). However, the effect of this treatment was found to be compromised when the patient harbors *H. pylori* isolates that are drug resistant (15, 16, 19). The prevalence of resistance to metronidazole has increased or remained steady over the past few years in several countries, including Singapore (3, 4, 6).

The disk diffusion test is routinely used to determine sensitivities to antibiotics, as it is economical and easy to perform (17). However, the agar dilution method, although tedious to perform, is still the reference method for the testing of antibiotic susceptibility (13). This method not only determines the antibiotic susceptibility but also the MICs for the bacteria. The commercially available E-test is a method that combines the advantages of the disk diffusion and agar dilution methods, as it is easy to use and also provides the MICs for the bacteria tested (5). However, the E-test is a commercial kit and for routine use is more costly than the agar dilution method, and studies have shown discrepancies in the results obtained by the two methods (1, 14).

In this study, we examined the relevance of metronidazole resistance in patients with PU and NUD, while the agar dilution method was used to determine the MICs for the *H. pylori* isolates.

### Materials and Methods

#### Processing of biopsy specimens

A total of 211 consecutive patients with dyspeptic symptoms were included in this study. Of these, 115 patients had PU, while 96 patients had NUD. PUs include duodenal or gastric ulcers, or both, while NUD is defined as dyspeptic symptoms but a lack of endoscopic evidence of an ulcer (27). The ages of the patients ranged from 17 to 86 years, with an average age of 45.7 years. Of the 211 patients, 133 were males and 78 were females. The study included 175 Chinese, 14 Malays, 14 Indians, and 8 patients of other ethnicities. Informed consent for gastroscopy and biopsy was obtained from all patients. Two biopsy specimens were taken from each patient. The specimens were obtained from the anterior and posterior gastric wall of the gastric antrum within 2 cm of the pylorus. The biopsy specimens were transported in 0.85% (wt/vol) sterile saline to the laboratory for processing within 8 h.

The biopsy specimens were smeared onto the surface of a selective chocolate blood agar and a nonselective chocolate blood agar, which were prepared by using Blood Agar Base No. 2 (Oxoid, Basingstoke, United Kingdom) supplemented with 5% heat-inactivated horse blood. The selective medium was supplemented with 10 \( \mu \)g of nalidixic acid (Sigma, St. Louis, Mo.) per ml, 5 \( \mu \)g of trimethoprim (Sigma) per ml, 3 \( \mu \)g of vancomycin (Sigma) per ml, and 2 \( \mu \)g of amphotericin B (Sigma) per ml. The plates were incubated microaerobically at 37°C in a humidified incubator (Forma Scientific, Marietta, Ohio) with 5% \( \text{CO}_2 \) for up to 14 days. The culture plates were checked for the presence of *H. pylori* on a daily basis. The bacterial strains isolated were confirmed to be *H. pylori* by Gram staining and the urease, oxidase, and catalase tests. The biopsy specimens were also examined by the rapid urease test (RUT) by the methods described by Vijayakumari et al. (23).
Metronidazole sensitivities of H. pylori isolates. The agar dilution method was carried out as described by Jorgensen et al. (9). Mueller-Hinton agar plates supplemented with 5% horse blood and twofold dilutions of metronidazole at concentrations ranging from 128 to 0.03 \mu g/ml were inoculated with 3-day-old cultures of different strains of H. pylori adjusted to a concentration of approximately 10^7 CFU/ml by using a multipoint inoculator (Denley, Billinghamurst, United Kingdom). All the plates were incubated at 37°C under microaerophilic conditions for 3 days in a humidified CO_2 incubator.

The MIC was defined as the lowest concentration of antibiotic that allowed no visible growth of the specific isolate on the antibiotic-containing plate. Bacterial strains were considered metronidazole resistant if the MIC was ≥8 \mu g/ml (10).

**UBT.** Patients with H. pylori infection were treated with a triple-therapy regimen for 7 days. The therapy comprised clarithromycin at 500 mg twice a day (b.i.d.) and metronidazole at 400 mg b.i.d. in combination with either lansoprazole at 30 mg b.i.d. or ranitidine-bismuth-citrate at 400 mg b.i.d. Patients who had a prior history of unsuccessful treatment were treated with quadruple treatment for 7 days. That treatment comprised lansoprazole at 30 mg b.i.d., tetracycline at 500 mg four times a day, metronidazole at 400 mg b.i.d., and ranitidine-bismuth-citrate at 400 mg b.i.d. A urea breath test (UBT) was performed 6 weeks after the completion of treatment. The patient was asked to drink 150 ml of a 0.1 N citric acid solution and to blow into a glass Exetainer test tube via a drinking straw to collect a baseline breath sample. The subject was then asked to swallow a 100-kg \textsuperscript{13}C-labeled urea tablet (Cambridge Isotope Laboratories, Cambridge, Mass.) with another 50 ml of the citric acid solution. Thirty minutes later, a second breath sample was collected in a second Exetainer test tube. Both breath specimens were analyzed for their \textsuperscript{13}C contents with a dedicated isotope ratio mass spectrophotometer (BreathMAT; Finnigan, Bremen, Germany). A change from the baseline value greater than 5% indicated active H. pylori infection.

**Statistical analysis.** Statistical analysis was carried out by Fisher’s exact test. A P value of less than 0.05 was used to represent a statistically significant difference.

**RESULTS**

**Culture and RUT of biopsy specimens.** H. pylori was isolated from 120 of 211 (56.9%) patients included in the study. Of these, 93 of 120 (77.5%) culture-positive specimens were cultured on both selective and nonselective media. Eighteen of them (15%) were cultured only on selective medium, while nine (7.5%) were cultured only on nonselective medium. The organism was successfully cultured from 81 of 115 (70.4%) PU patients and 39 of 96 (40.6%) NUD patients. H. pylori was isolated from 78 of 133 (58.6%) male patients, while 42 of 78 (53.8%) females harbored the bacterium. Of the 175 Chinese included in the study, 96 (54.9%) were found to be culture positive, while 10 of 14 (71.4%) Malays, 10 of 14 (71.4%) Indians, and 4 of 8 (50%) patients of other ethnicities were infected with H. pylori.

The percentage of culture-positive specimens was 56.9% (120 of 211), while a positive RUT result was observed in 57.8% (122 of 211) of the specimens.

**Prevalence of metronidazole resistance.** Of the 120 H. pylori isolates, 38 (31.7%) were found to be metronidazole resistant (MICs, ≥8 \mu g/ml) by the agar dilution method. The MICs ranged from ≤0.03 to ≥128 \mu g/ml. The MICs at which 50 and 90% of the isolates were inhibited were 4 and 64 \mu g/ml, respectively.

The results showed no correlation between the metronidazole susceptibilities of the H. pylori isolates and patient age, irrespective of the clinical diagnosis. Similarly, there was also no significant difference between patient gender and the prevalence of metronidazole-resistant H. pylori (23 of 78 [29.5%] males and 15 of 42 [35.7%] females harbored resistant strains; P = 0.54) (Table 1).

The prevalence of metronidazole resistance was also not found to be associated with the ethnicity of the patient in the local multiethnic population (P > 0.05). Of the 96 H. pylori-positive Chinese patients, 32 (33.3%) were found to harbor metronidazole-resistant H. pylori strains, while 3 of 10 (30%), 1 of 10 (10%), and 2 of 4 (50%) of Malay, Indian, and patients of other ethnicities, respectively, were found to harbor metronidazole-resistant strains (Table 1). However, significantly (P < 0.001) more H. pylori-positive NUD patients (22 of 39 [56.4%]) than PU patients (16 of 81 [19.8%]) were found to harbor metronidazole-resistant bacteria (Table 1). A similar trend was observed between the PU and NUD patients of Chinese origin. Among 96 H. pylori-positive Chinese patients, significantly more (P < 0.001) NUD patients (18 of 30 [60%]) than PU patients (14 of 66 [21.2%]) harbored metronidazole-resistant H. pylori strains. Although the numbers of patients of the other ethnicities were too small for statistical analysis, the results demonstrated that more NUD patients than PU patients were infected with metronidazole-resistant H. pylori strains (Table 1).

**UBT.** Table 2 shows that of the 81 H. pylori-positive patients with a follow-up UBT 6 weeks after treatment, 75 (92.6%) were H. pylori negative on the basis of UBT guidelines (25). Negative UBT results were recorded for 62 of 63 (98.4%) and 13 of 18 (72.2%) PU and NUD patients, respectively. Among the six patients with positive UBT results (indicative of treatment failure [25]), four had NUD and harbored metronidazole-resistant H. pylori isolates, and the isolates from one of these patients were also resistant to clarithromycin. This is in contrast to the negative UBT results (successful eradication

### Table 1. Prevalence of metronidazole-resistant H. pylori

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>Metronidazole sensitivity</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gender</td>
<td>Female</td>
</tr>
<tr>
<td>PU</td>
<td>S</td>
<td>19</td>
</tr>
<tr>
<td>NUD</td>
<td>S</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>8</td>
</tr>
</tbody>
</table>

**Metronidazole sensitivity results are based on the results of the agar dilution method (9). S, sensitive; R, resistant.**

### Table 2. Posttreatment UBT results

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>R, metronidazole resistant; S, metronidazole sensitive.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Successful</td>
</tr>
<tr>
<td>PU</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>S</td>
</tr>
<tr>
<td>NUD</td>
<td>R</td>
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<tr>
<td></td>
<td>S</td>
</tr>
<tr>
<td>Total</td>
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**The values in parentheses indicate the number of patients harboring clarithromycin-resistant strains.**
The present study were placed into the PU and NUD groups according to the age, gender, or ethnicity of the patient (Table 1). This is especially so with the increased rates of antibiotic resistance among H. pylori isolates (3, 4, 6). The present study shows that the use of a combination of selective and nonselective media could facilitate the successful isolation of H. pylori from gastric biopsy specimens, as was described by Hua et al. (7). The percentage of culture-positive specimens (56.9%) was comparable to the percentage of RUT-positive gastric biopsy specimens (57.8%) (P > 0.05). Since the RUT result is available within 4 h, the test provides useful preliminary information on the isolation of H. pylori. Thus, use of the combination of culture and RUT is desirable in the diagnosis of H. pylori infection, as was reported earlier (7, 26).

The MICs based on the results of the agar dilution method showed that there was no correlation between the presence of a metronidazole-resistant H. pylori strain and the age, gender, or ethnicity of the patient (Table 1). The patients in the present study were placed into the PU and NUD groups according to the clinical diagnosis and gastroscopy findings. The MICs indicated that metronidazole-resistant H. pylori strains were almost threefold more frequent in the NUD patients (22 of 39 [56.4%]) than in the PU patients (16 of 81 [19.8%]) (P < 0.001) (Table 1). This significantly higher prevalence of metronidazole resistance among isolates from patients with NUD was also observed regardless of the ethnicities of the patients.

This result contradicts those observed in several studies that showed a higher percentage of metronidazole-resistant H. pylori strains in patients with PU (24) or no difference in the prevalence of metronidazole-resistant strains from NUD and PU patients (22, 26). However, the patients involved in our study were of Asian origin, whereas the three studies (22, 24, 26) whose results contradict those of our study were carried out with Western populations. The differences in metronidazole resistance observed could probably be due to the frequent use of metronidazole for the treatment of parasitic and periodontal diseases in Asia, as was reported earlier (12).

In a separate study with Asians by Ching et al. (3), there was a twofold increase in the numbers of metronidazole-resistant H. pylori strains recovered from asymptomatic controls than from PU patients. However, the present study shows an even higher (approximately threefold) prevalence of metronidazole-resistant H. pylori strains in NUD patients than in PU patients. Both studies concurred that metronidazole-resistant H. pylori is more prevalent in the non-PU patients. It further implies that patients in an Asian population without PUs are more likely to harbor metronidazole-resistant H. pylori strains. The difference between the two studies rested on a comparison of asymptomatic subjects (healthy volunteers) and PU patients (3), while the present findings were based on a controlled study with consecutive patients with dyspeptic symptoms (patients with NUD and patients with PU). However, in another study with asymptomatic subjects, Vaira et al. (21) showed that of 128 of 1,010 asymptomatic blood donors who were seropositive and who had consented to endoscopy, 121 had chronic active gastritis. Among these 121 patients, 25 had PU, 21 had erosive duodenitis, and 2 had gastric cancer. The investigators concluded that H. pylori infection, even if it is asymptomatic, is of far greater clinical relevance than was originally thought.

In the present study, 39 H. pylori-positive patients did not return for follow-up for the posttreatment UBT. Of the 81 patients who had a follow-up UBT, 4 of 6 (66.7%) subjects who had a positive UBT result (failed treatment) were NUD patients infected with metronidazole-resistant H. pylori. On the contrary, none of the 13 PU patients who harbored metronidazole-resistant H. pylori strains failed treatment. This further indicates the strong association of metronidazole-resistant H. pylori and NUD. Of the remaining two patients with treatment failure (positive UBT results), one had PU and was infected with an H. pylori strain for which the MIC was 4 μg/ml (a value close to the cutoff for metronidazole resistance), while the other patient had NUD and was treated with an H2 antagonist.

The present study provides evidence of a higher prevalence of metronidazole resistance in patients with NUD than in those with PU. Further studies are needed to clarify the underlying reasons for this. One possible hypothesis is that the different physiological environments of the stomachs of the respective patients, such as the lower degree of damage to the gastric epithelium and the mucous layer in the stomachs of patients without ulcers, may provide protection to the colonizing H. pylori isolates. This may result in a reduced level of accessibility of antibiotics to the H. pylori isolates that reside below the mucous layer, leading to the exposure of the bacteria to suboptimal antibiotic concentrations, which could possibly result in the induction of metronidazole resistance of the H. pylori isolates in patients with NUD. This phenomenon has been demonstrated in an in vivo study (8) in which metronidazole-resistant H. pylori strains were readily selected by prior exposure of mice to sublethal doses of metronidazole. Similarly, it was shown in another study (20) that the selection of metronidazole-resistant H. pylori strains in vitro could be achieved by serial passage of the bacteria on plates containing subinhibitory concentrations of metronidazole. Thus, the high prevalence of metronidazole-resistant H. pylori in Asian patients with NUD is of concern. It may be useful to reexamine the use of metronidazole in the treatment of NUD patients harboring metronidazole-resistant H. pylori strains.

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