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Susceptibility Testing of *Helicobacter pylori* to Metronidazole and DNA Fingerprints of Resistant Strains in Singapore

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Abstract Susceptibility of 61 strains of *Helicobacter pylori* to metronidazole was examined by both the disk diffusion method using a cut-off of 15 mm for resistance and the E test with a cut-off of 8 mg/l. The MIC_{50} and MIC_{90} by the E test were 2 mg/l and 256 mg/l, respectively. Metronidazole resistance was found in 22 (36%) out of the 61 *H. pylori* strains by the E test and in three additional strains by the disk diffusion method. Amongst the latter three isolates, the MICs by the E test were 4 mg/l, 6 mg/l, and 6 mg/l, respectively. These figures are one $\log_2$ or half $\log_2$ dilution lower than the cut-off of 8 mg/l recommended as resistance for the E test. All 22 metronidazole resistant *H. pylori* isolates by the E test that were subjected to random amplified polymorphic DNA (RAPD) fingerprinting showed different DNA fingerprints. Interestingly, >90% of resistant isolates possess two common DNA bands of 0.4 and 0.9 kb. This study demonstrates that the results of the disk diffusion method for testing *H. pylori* susceptibility to metronidazole correlates well with that of the E test. The criteria for interpretation need to be internationally standardized so that the results from different centers can be compared.

Key words: Antibiotic susceptibility testing, *Helicobacter pylori*, metronidazole, disk diffusion method, E test, DNA fingerprinting

Eradication of *Helicobacter pylori*, a gram-negative bacterium which infects the human stomach, has a positive effect in curing peptic ulcer disease and reduces the frequency of ulcer relapse [13, 23]. Current treatment regimens consist of combinations of either a proton pump inhibitor or bismuth compound together with two antibiotics [6, 20]. Metronidazole is one of the two popularly used antibiotics, due to its cost and efficiency. It has been reported that patients infected with metronidazole-resistant strains of *H. pylori* have a lower eradication rate than patients infected with metronidazole-susceptible strains [5, 17, 21]. Therefore, it is crucial in determining the local prevalence of antibiotic resistance for the purpose of guiding the selection of an appropriate treatment regimen. Susceptibility testing of *H. pylori* is not routinely performed in the Asia Pacific region [14]. The differences in methodology and interpretation of results have contributed to the difficulty in making comparison of studies from different centers and countries [4, 5, 15, 18, 26]. The present study was performed to directly compare the *in vitro* susceptibility of *H. pylori* to metronidazole using the disk diffusion and E test methods. Subsequently, the DNA fingerprints of metronidazole-resistant strains of *H. pylori* were also examined

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Materials and Methods

Bacterial Strains

H. pylori was isolated from 61 gastric biopsy specimens obtained from patients endoscoped for dyspepsia from the period of 1995 to 1997 at the National University Hospital in Singapore. The patients comprised of 25 female and 36 male subjects with a mean age of 55 years (range 19 to 84 years). Of 61 strains, 38 were isolated from patients with duodenal ulcer, 16 from patients with gastric ulcer, 3 from patients with gastric cancer, and 4 from patients with non-ulcer dyspepsia. None of the 61 patients had been previously treated for *H. pylori* or had known exposure to antibiotics, bismuth compound, or proton pump inhibitor in the past four weeks. Two gastric biopsies were obtained from the gastric antrum within 2 cm of the pylorus during upper endoscopy and transported in sterile saline to the microbiological laboratory within 24 h. Specimens were smeared onto a horse chocolate blood agar plate (HCBA) without antibiotic and then onto another HCBA plate supplemented with antibiotics (vancomycin 3 mg/l, colistin methane sulphate 7.5 mg/l, nystatin 12,500 U/l, and trimethoprim 5 mg/l). The plates were incubated at 37°C

in an atmosphere of 5% CO_2. Cultures were examined up to 10 days of incubation. *H. pylori* isolates from positive cultures were identified by Gram staining for catalase, oxidase, urease reactions, and API ZYM test [11]. The organisms were maintained at -80°C in aliquots of 1 ml of brain heart infusion broth supplemented with 20% glycerol and 10% horse serum. For subsequent analysis, the bacteria were subcultured on HCBA at 37°C in 5% CO_2 atmosphere for 72 h. *H. pylori* NCTC 11637, a standard strain, was also incorporated into the study for susceptibility testing and DNA fingerprint analysis.

Antibiotic Susceptibility Testing

The disk diffusion test and E test were used for the purpose of testing the bacterial sensitivity to antimicrobial agents. An inoculum of *H. pylori* suspension equivalent to McFarland 3 turbidity standard was spread onto the HCBA plate. The plates were allowed to dry for 5-10 min before metronidazole disks (6 mm disk containing 5 µg per disk, Oxoid) or E test strips (range 0.016-256 µg/ml, AB BIODIK) were placed. These HCBA plates were incubated at 37°C in 5% CO_2 atmosphere for 2-4 days.

Genomic DNA Extraction

Plate culture of *H. pylori* was transferred into an Eppendorf tube and 1.5 ml volume of TE buffer (100 mM Tris-HCl and 1 mM EDTA, pH 8.0) was added. The suspension was centrifuged at 8000×g and washed once with TE buffer. The pellet was suspended in 800 µl TE buffer. The bacterial suspension was incubated in 100 µl of 10 mg/ml lysozyme (Sigma) at 37°C for 30 min, and then lysed with 100 µl of 10% sodium dodecyl sulfate for an additional 30 min at 37°C. Following the addition of 5 µl of 10 mg/ml proteinase K (Boehringer Mannheim), the mixture was incubated for 1 h at 56°C. DNA was purified by extracting twice with an equal volume of phenol and once with an equal volume of chloroform. DNA was then precipitated overnight with two volumes of absolute ethanol and 1/10 volume of 3 M sodium acetate at -20°C. The DNA precipitate was washed once with 70% ethanol [10]. The pellet was vacuum-dried using a speed-vac (Savant) and resuspended in 200 µl sterile distilled water. This served as target DNA for PCR. DNA concentration was measured spectrophotometrically at 260 nm.

Random Amplified Polymorphic DNA (RAPD) Fingerprinting

The universal primer for PCR-based RAPD was randomly chosen according to Akopyanz *et al.* [1] which allows for the fingerprinting of the whole DNA content of cells. The primer used in this study was 5-AAGAGCCCGT-3. PCR reaction was carried out in 25 µl volume. Fifty nanogram of *H. pylori* genomic DNA, 20 pmol of primer, 1 unit of Taq DNA polymerase, and 250 µM each of dGTP, dCTP, dATP, and dTTP were placed in standard PCR incubation buffer containing 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, and 0.01% gelatin (Promega, U.S.A.). The reaction mixture was overlaid with a drop of mineral oil to prevent evaporation. PCR was performed with a thermal cycler (Amplitron, U.S.A.) consisting of an initial step of denaturation of target DNA at 94°C for 5 min. This procedure was followed by 39 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min. Ten microliters of the PCR products were electrophoresed in 1% horizontal agarose gels for 2 h at 80 V in TBE buffer. The gels were stained with ethidium bromide (1 µg/ml) and photographed with a filtered UV illumination on Polaroid type 667 film.

Statistical Analysis

MIC_{50}, MIC_{90}, linear regression, and regression coefficient were calculated. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

A total of 61 *H. pylori* strains were tested by both the disk diffusion method and E test (Table 1). The correlation between the MICs and the inhibition zone diameters is showed in Fig. 1. The regression coefficient was 0.82 and there was a statistically significant correlation between the disk diffusion method and E test ($P<0.05$). MIC_{50} and MIC_{90} were 2 mg/l and 256 mg/l by the E test, respectively. In the E test, strains with a MIC >8 mg/l were regarded as resistant to metronidazole, while in the disk diffusion method a cut off with an inhibition zone diameter of less than 15 mm was chosen as resistance according to DeCross *et al.* [5]. Metronidazole resistance was found in 22 (36%) of the 61 *H. pylori* strains by the E test (Table 1). The disk diffusion method defined 3 more resistant strains with inhibition zone diameters <15 mm as compared to the E test. Amongst these three isolates, one was with MIC of 4 mg/l and had the inhibition zone diameter of 12 mm, while the other two isolates with MICs of 6 mg/l showed the inhibition zone diameters of 6 mm and 13 mm, respectively. No statistical difference was found between the disk diffusion method and E test ($P>0.05$).

DNA fingerprints of 22 metronidazole resistant *H. pylori* strains with MIC levels ≥8 mg/l by the E test were examined by RAPD. All these 22 metronidazole resistant strains showed different RAPD fingerprinting patterns (Fig. 2). Each strain could be easily differentiated from one another by comparing their DNA fingerprints. No specific pattern was found to be associated with metronidazole resistance. However, it was interesting to note that two bands of 0.4 and 0.9 kb were present in

Table 1. Comparison between the disk diffusion method and E test for metronidazole susceptibility testing of *H. pylori*.

Number of strains (Total 61)	E test MIC (μg/ml)	Disk diffusion zone (mm)
9	>256	6
1	128	6
1	96	6
1	64	6
1	32	6
1	12	6
2	8	6
1	6	6
1	12	8
1	10	8
1	64	11
1	4	12
1	6	13
1	96	14
1	32	14
1	8	15
1	6	16
1	4	16
1	4	17
1	2	17
1	0.5	19
1	0.125	20
1	1	21
1	4	22
1	1.5	22
1	1	22
1	0.5	22
1	2	23
1	0.75	23
1	0.094	24
1	4	25
1	0.75	25
1	0.38	25
2	0.25	25
1	0.125	25
1	1	26
1	0.5	26
1	0.38	27
1	0.25	27
1	1	31
1	0.5	31
1	0.75	34
1	0.19	35
1	0.38	36
2	0.19	36
1	0.5	37
2	0.75	38
1	0.125	39
1	0.064	65

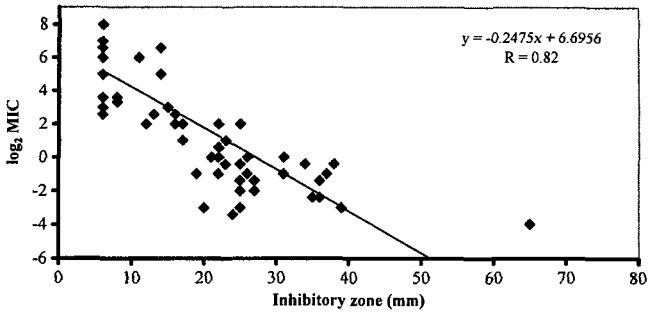

Fig. 1. Correlation between inhibition zone diameter and MIC (n=61).

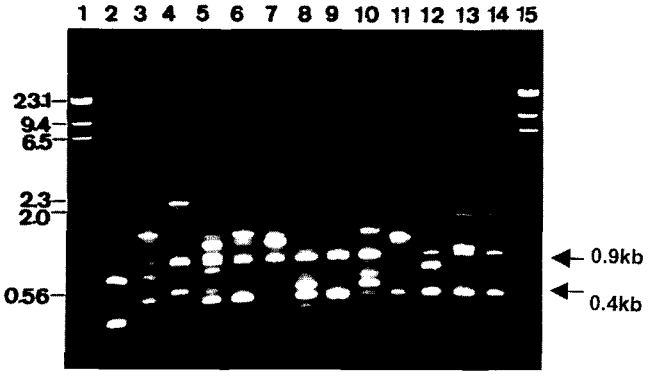

Fig. 2. Representative results of RAPD fingerprints of different *H. pylori* isolates. PCR products were run on 1% agarose gel. The two common bands of 0.4 and 0.9 kb were present in the majority of metronidazole resistant strains. Lanes 1 & 15, λ DNA digested with *Hin*dIII; Lane 2, NCTC 11637, metronidazole sensitive; Lanes 3-14, metronidazole resistant strains.

DNA fingerprints produced by 90% (20/22) and 95% (21/22) of metronidazole resistant strains, respectively, but were absent in a metronidazole sensitive strain, NCTC 11637.

DISCUSSION

H. pylori infection is relatively difficult to treat although this bacterium is sensitive to many antibiotics in *in vitro* conditions [22]. Metronidazole is one of the most effective and widely used drugs in combination therapy to eradicate *H. pylori*. However, the effectiveness of the combination treatment of gastric infection caused by *H. pylori* has been known to be reduced by the organisms' resistance to metronidazole [5, 17, 21]. This has made susceptibility testing of *H. pylori* increasingly important in clinical practice. Different methods, including broth microdilution, agar dilution, disk diffusion methods, and E test, have been used for susceptibility testing for *H. pylori* [5, 19, 22, 26]. These methods generally perform well but have their respective limitations. The broth microdilution method is limited by the difficulty in growing *H. pylori* in liquid

media along with the possibility of contamination due to prolonged incubation. The agar dilution method seems to be satisfactory when large numbers of strains are tested simultaneously. However, since this method is labor-intensive and costly for determining the MIC of a single strain, it is not routinely used in a clinical laboratory. An excellent agreement has been reported between the agar dilution method and E test [19]. The basis of the latter test is a plastic strip that incorporates an antibiotic gradient consisting of two folded dilution steps as well as half steps. The drug diffuses from the strip into the agar and, by doing so, it effectively forms a gradient with the bilateral symmetry. The MIC value is the exact point where the inhibition zone meets with the border of the strip. However, use of the E test in developing countries is limited owing of its overwhelming cost.

In this study, the susceptibility of 61 clinical isolates of *H. pylori* to metronidazole was examined by using both the disk diffusion method and the E test. Metronidazole resistance was found in 22 (36%) of the 61 *H. pylori* strains by the E test. The disk diffusion method with a cut-off value of 15 mm showed three additional resistant isolates. Although the disk diffusion method is known to be cost-effective and easy to perform, it does not provide an actual MIC and the cut-off value has not been well standardized. Several investigators have tried using this method to test *H. pylori* sensitivity to metronidazole. Owen *et al.* [18] arbitrarily chose a zone of inhibition of more than 10 mm as indicating susceptibility. DeCross *et al.* [5] compared the disk diffusion method with Steer's replication agar dilution procedure and found that the correlation between the MIC and zone diameters was generally agreeable. They used a zone diameter of more than 15 mm as criteria for susceptible isolates. Xia *et al.* [26] proposed three interpretative categories of susceptibility. Strains with zone diameters of less than 20 mm should be reported as resistant, while those with zone diameters of 20 to 26 mm should be reported as intermediate, and finally those with zone diameters of greater than 26 mm should be reported as susceptible. In these three separate studies, a 6-mm-diameter disk containing 5 μg of metronidazole was applied in the disk diffusion method. However, the difference in media and condition of incubation used might account for the discrepancy of the criteria that was applied by different investigators. In 1996, Ching *et al.* [4] reported that the overall metronidazole resistance rate was 53.5% (37 of 69) among peptic ulcer disease patients and normal controls in Hong Kong when the inhibition zone diameter of less than 15 mm was regarded as resistant to metronidazole. Another separate report [15] made from Hong Kong showed that the prevalence of metronidazole-resistant strains rose rapidly from 22.0% (29 of 132) in 1991 to 73.2% (197 of 269) in 1995. However, in the latter study, the authors used three interpretative categories as proposed by Xia *et al.* [26]. It would be difficult to compare the results of susceptibility of *H. pylori* to metronidazole from different reports due to discrepancy of the criteria despite the fact that these investigators have studied the subjects from the same region. Therefore, standardization and consensus in the interpretation of the disk diffusion method are needed to make an effective comparison study.

In this study, a cut-off of 15 mm for the disk diffusion method was applied according to DeCross *et al.* [5]. The results of the disk diffusion method and E test correlated well (r=0.82) and were consistent with the findings of DeCross *et al.* [5]. A discrepancy was noted in 3 strains. The disk diffusion method showed three false resistant cases when the E test was used as a reference. However, the MICs of these three isolates were in the borderline and only one $\log_2$ or half $\log_2$ dilution lower than the cut-off of 8 mg/l which is recommended as resistance to metronidazole for the E test. Although it is generally well known that the E test represents an excellent alternative, reproducible method for assessing the susceptibility of *H. pylori* to antimicrobial agents, it also needs to be mentioned that underestimation of MICs by the E test has been described in some studies [9, 19]. Among 71 clinical isolates of *H. pylori*, Piccolomini *et al.* [19] observed 8 errors with metronidazole in 20 antimicrobial agents tested. As a result, some investigators [2] suggested that the results obtained by the E test with metronidazole should be confirmed by the agar dilution method.

The exact mechanism of resistance of *H. pylori* to metronidazole is not well known. Edwards [7] suggested the possible mechanism of induction of superoxide dismutase and catalase by bacteria which prevent hydroxyl ion formation along with the increase in free radical scavenging compounds, thus minimizing oxidative damage. Since *H. pylori* can acquire resistance to metronidazole by natural transformation [12, 16, 25], a genetic mechanism is also suspected. Chang *et al.* [3] found that mutation in the *recA* gene might be associated with metronidazole resistance of *H. pylori*. Recently, Goodwin *et al.* [8] demonstrated that metronidazole resistance in *H. pylori* is caused by null mutations in a gene, *rdxA*, which resulted in loss of oxygen-insensitive NADPH nitroreductase activity. This study examined the DNA fingerprints of all resistant isolates by RAPD and demonstrated that these isolates are genotypically heterogeneous and can be distinguished from each other, suggesting that they do not originate from a single source. However, it is interesting to note that two bands of 0.4 and 0.9 kb were present in DNA fingerprints of 90% and 95% metronidazole-resistant strains, respectively, but were absent in NCTC 11637 which is sensitive to metronidazole. Further study is needed to verify whether or not these two bands are absent in all clinical metronidazole-sensitive strains. The nature

of these two bands could possibly be identified with the use of the genomic library of *H. pylori* published [24].

In conclusion, the disk diffusion method could be used effectively for testing the susceptibility of *H. pylori* to metronidazole as it is easy to perform and cost-effective. Furthermore, it also correlates well with the E test. It is imperative that the protocols and criteria for interpretation for the disk diffusion test need to be internationally standardized so that results from different centers can be readily compared.

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