Standardization of Disk Diffusion Test and Its Clinical Significance for Susceptibility Testing of Metronidazole against Helicobacter pylori

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Susceptibilities of 121 clinical Helicobacter pylori strains to metronidazole were determined by both a 5-μg metronidazole disk diffusion test and a plate dilution method in duplicate and after different periods of incubation. The distribution of MICs of metronidazole against H. pylori among the strains was found to be bimodal. The diameters of inhibitory zones obtained by the disk diffusion test and the MICs obtained by the plate dilution method correlated well, especially after 4 days of incubation (r = 0.77). An inhibitory zone diameter of 20 mm was found to correspond to a MIC of 8 μg/ml and is recommended as a suitable zone for differentiating susceptibility and resistance with a 5-μg metronidazole disk. Three interpretive categories of susceptibility results were defined; strains with inhibitory zone diameters of more than 26 mm were defined as susceptible (MIC, <4 μg/ml), strains with zone diameters of 20 to 26 mm were deemed intermediate (MIC, 4 to 8 μg/ml), and those with zone diameters of less than 20 mm were deemed resistant (MIC, >8 μg/ml). Furthermore, 76 H. pylori-positive patients with duodenal ulcers or nonulcer dyspepsia were treated with a 1 week of triple therapy (colloidal bismuth subcitrate, metronidazole, and tetracycline). H. pylori strains were isolated before treatment from antral biopsies from those patients, and the metronidazole susceptibilities of the strains were determined by the disk diffusion test. H. pylori status was evaluated again 4 weeks after completion of treatment. The eradication rates for susceptible, intermediate, and resistant strains were 95.9% (47 of 49), 62.5% (5 of 8), and 52.6% (10 of 19), respectively. It is concluded that the 5-μg disk diffusion test is easy to perform and gives final results similar to those of the plate dilution method. The three interpretive categories of susceptibility may be of benefit for chemical choice of chemotherapy in eradicating H. pylori.

Nitroimidazoles, including metronidazole and tinidazole, have been used in multiple antimicrobial therapy strategies to eradicate Helicobacter pylori in peptic ulcer patients and nonulcer dyspeptic patients (1, 19). However, emergence of primary resistance and acquired resistance to these kind of drugs in H. pylori may lead to treatment failure (3, 7, 10, 11, 21, 27). Thus, evaluation of H. pylori susceptibility to metronidazole before treatment appears to be important. Metronidazole resistance rates from 6.4 to 84% have been reported throughout the world (2, 11, 13, 22). In Ireland, 27.5% of pretreatment strains of H. pylori were resistant to metronidazole (26). In other European countries, the resistance rate varied from 7 to 49% (8). The difference in the rates reported may reflect the variation in metronidazole usage between countries, since use of metronidazole alone can easily induce resistance to the drug in H. pylori (11, 14, 24). Furthermore, different susceptibility testing methods and interpretation of their results may also contribute to the varied resistance rates. Therefore, standardized testing of the metronidazole susceptibility of H. pylori is important and simple, accurate, and precise methods are required for routine use. The aims of the present study were (i) to determine the distribution of the MICs of metronidazole against clinical H. pylori strains by a plate dilution method, (ii) to establish a performance and interpretation system for a 5-μg metronidazole disk diffusion test by comparing the diameters of inhibitory zones obtained by the disk diffusion test with the MICs obtained by the standard plate dilution method, and (iii) to evaluate the clinical applicability of the disk diffusion test and of three interpretive categories of susceptibility results (i.e., susceptible, intermediate, and resistant).

MATERIALS AND METHODS

Preparation of bacterial cells. One hundred twenty-one clinical strains of H. pylori isolated from antral biopsy specimens from 121 dyspeptic patients and reference strain NCTC 11639 (from the National Collection of Type Cultures, Public Health Laboratory, London, England) were subcultured onto chocolate agar plates (Columbia agar base with heated 7% horse blood) and incubated under microaerophilic conditions at 37°C for 3 to 4 days. A subculture of each strain was suspended in 2 ml of nutrient broth and then adjusted to a turbidity equivalent to a McFarland no. 3 standard, yielding a viable count of 106 to 109 CFU/ml (8, 28). This suspension density is necessary since H. pylori is very fastidious and the resultant colonies on solid agar are tiny (7, 28).

Susceptibility testing. An inoculum of 1 μl of each suspension (108 to 106 CFU) was transferred onto the surface of Columbia blood agar (with 7% horse blood) containing metronidazole (Sigma Chemical Co., St. Louis, Mo.) in concentrations ranging from 0.5 to 64 μg/ml by using a multipoint inoculator (Biddulph Co. Ltd., Manchester, United Kingdom). Sterile swabs were dipped into the original suspensions (106 to 108 CFU/ml) of each strain and applied to blood plates without antibiotics, and a 6-mm-diameter disk containing 5 μg of metronidazole (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) was applied to each plate with a disk dispenser (Oxoid). The plate dilution methods and the disk diffusion tests were each performed in duplicate. All plates were incubated under the conditions described above. MICs and diameters of inhibitory zones were read at 3, 4, 5, and 6 or 7 days.
To check the reproducibility of the two techniques, metronidazole-susceptible reference strain NCTC 11639, with a predetermined metronidazole MIC of 1 μg/ml (range, 0.5 to 1 μg/ml) and an inhibitory zone diameter of 40 mm (range, 36 to 44 mm), was used as a control for the first 50 strains.

**Definition and interpretation of susceptibility testing results.** The MIC was defined as the lowest concentration of metronidazole at which there was no visible growth of *H. pylori* on agar after given periods of incubation (26). In the disk diffusion test, diameters of inhibitory zones were recorded as 6 mm when the edge of the disk was occupied by colonies of the organism. When separate or individual colonies (mostly resistant mutants) occurred within an apparent inhibitory zone, the colonies closest to the disk determined the zone size. When two MICs obtained for one strain were different (19.8% of the strains tested), the higher MIC was taken as the MIC for that strain, while a mean value of two diameters of inhibitory zones for a strain was taken as the diameter of the inhibitory zone for that strain.

A MIC of 8 μg/ml has been used in previous studies as a breakpoint to differentiate susceptible and resistant strains (11, 13, 18, 27). In the present study, three interpretive categories were defined; strains with metronidazole MICs of less than 4 μg/ml were defined as susceptible, strains with metronidazole MICs between 4 and 8 μg/ml were defined as intermediate, and those with metronidazole MICs of greater than 8 μg/ml were deemed resistant, since strains with metronidazole MICs between 4 and 8 μg/ml can be more easily selected to become metronidazole resistant (24). In the disk diffusion test, strains with diameters of inhibitory zones corresponding to the MICs described above were defined as susceptible, intermediate, and resistant, respectively, after the correlation between the diameters of inhibitory zones obtained by the disk diffusion test and MICs obtained by the agar dilution method had been assessed.

**Clinical trials.** Subsequently, 76 consecutive patients, 45 female and 31 male, with a mean age 48.3 years (range, 16 to 79 years) were included in a clinical study. Sixty were diagnosed as having duodenal ulcers, and 16 had nonulcer dyspepsia at endoscopy. *H. pylori* strains were isolated from antral biopsy specimens from these patients, and the susceptibilities of these strains to metronidazole were determined by using the disk diffusion test (4 days of incubation). The patients were treated with colloidal bismuth subcitrate (120 mg four times a day) for 4 weeks and metronidazole (400 mg three times a day), and tetracycline (500 mg three times a day) for the first week without knowledge of laboratory results. Compliance was monitored by tablet counts, and all of the patients took more than 95% of the prescribed medications. Endoscopy was repeated at least 4 weeks after completion of the treatment, and *H. pylori* status was determined by rapid urease test (CLO test; Delta West Pty Ltd., Bentley, Western Australia) and histological (hematoxylin and eosin stain) and microbiological (Gram stain and culture) procedures. The metronidazole susceptibility of nonradiated *H. pylori* strains (if isolated) was also determined by the disk diffusion test. Since *H. pylori* is always susceptible to tetracycline, before and after treatment with the drug (27), tetracycline susceptibility testing was not performed in this study.

**Statistical method.** Logarithmic regression analysis was used to establish the correlation between the diameters of inhibitory zones obtained by the disk diffusion test and MICs obtained by the agar dilution method. Difference in the efficacy of the triple therapy in patients with susceptible strains and those with resistant strains was assessed with the chi-square test, and the differences between patients with susceptible, intermediate, and resistant strains were assessed with the chi-square test with Yates's correction.

**RESULTS**

**Testing of *H. pylori* susceptibility to metronidazole in vitro.** Both the agar dilution and disk diffusion methods gave reasonable agreement in the duplicate tests. With the dilution method, 97 (80.2%) of the 121 strains tested achieved the same MICs in the duplicate tests and the differences for the other strains (19.8%) were within acceptable limits (1 twofold dilution). With the disk diffusion test, 103 strains (85.1%) gave identical inhibitory zone diameters in the two tests while the maximum difference for the others (14.9%) was never more than 10 mm.

Among the 121 clinical *H. pylori* strains tested, 88 (72.7%) were metronidazole susceptible, with metronidazole MICs of less than 4 μg/ml; 9 (7.4%) were intermediate, with MICs between 4 and 8 μg/ml; and 24 (19.8%) were resistant, with MICs of greater than 8 μg/ml. The distribution of metronidazole MICs for 121 clinical *H. pylori* strains after 4 days of incubation is shown in Fig. 1.

**Comparison of the disk diffusion test with the agar dilution method.** By comparison of diameters of inhibitory zones obtained by the disk diffusion test with MICs obtained by the agar dilution method, it was found that 4 days of incubation achieved the best correlation coefficient ($r = 0.77$) while 5 and 3 days of incubation were less satisfactory ($r = 0.75$ and $r = 0.73$, respectively). Incubation for 6 to 7 days further decreased the correlation coefficient ($r = 0.72$).

By using the regression equation (zone diameter = 36.35 – 5.54 log$_2$ MIC) obtained after 4 days of incubation, the diameters corresponding to MICs of 4 and 8 μg/ml were calculated to be 25.27 and 19.73 mm, which, for practical purposes, were rounded to 26 and 20 mm, respectively. Thus, a diameter of 20 mm is recommended as the zone size which

![FIG. 1. Distribution of the MICs of metronidazole against 121 *H. pylori* strains.](http://aac.asm.org/)
might differentiate between susceptibility and resistance. Correspondingly, strains with zone diameters of 20 to 26 mm were reported as intermediate while strains with zones of greater than 26 mm were reported as susceptible and those with the zones of less than 20 mm were reported as resistant when three interpretive categories were used. By using these criteria in the present study, 81 (67%) of the 121 strains tested were reported as susceptible, 12 (9.9%) were reported as intermediate, and 28 (23.1%) were reported as resistant when incubation was done for 4 days. The total interpretive error rate was 10.7% (13 of 121). Two susceptible strains (with a MIC of 2 μg/ml) and three intermediate strains (one with a MIC of 4 μg/ml and two with a MIC of 8 μg/ml) were incorrectly reported as resistant, while one resistant strain (with a MIC of 32 μg/ml) was incorrectly reported as intermediate (with a zone diameter of 24 mm). Six susceptible strains (with MICs between 1 and 2 μg/ml) were reported as intermediate, and another intermediate strain (with a MIC of 4 μg/ml) was reported as susceptible (Fig. 2). However, the false-susceptibility rate was 0% (0 of 24) and the false-resistance rate was 2.3% (2 of 88). Results obtained after incubation for 3 days showed a slightly higher interpretive error rate of 14.9% (18 of 121) and a false-resistance rate of 3.4% (3 of 88), while data obtained after incubation for 5 and 6 or 7 days showed interpretive error rates similar and false-resistance rates identical to those obtained after 4 days of incubation (data not shown). Thus, the best agreement between the two methods was achieved when a zone of less than 20 mm represented resistance and a zone of greater than 26 mm represented susceptibility.

Outcome of clinical trials. Among the 76 strains isolated from patients before treatment with triple therapy, 51 were metronidazole susceptible, 6 were intermediate, and 19 were resistant, as determined by the disk diffusion test. The triple therapy eradicated the H. pylori from 62 patients (81.6%). The clinical eradication rate for laboratory-susceptible strains was 91.2% (52 of 57), while the rate for resistant strains was 52.6% (10 of 19) (P < 0.001). With the three interpretive categories of susceptibility, the eradication rates for susceptible, intermediate, and resistant strains were 95.9% (47 of 49), 62.5% (5 of 8), and 52.6% (10 of 19), respectively. The difference between the eradication rates of susceptible and intermediate strains was significant (P < 0.02), while there was no significant difference between those of intermediate and resistant strains.

Strains of H. pylori were isolated from 11 of the 14 patients who had persistent H. pylori infections. All of these strains, including five originally susceptible or intermediate strains, were resistant to metronidazole.

DISCUSSION

Metronidazole is one of the most successful drugs used in combination to eradicate H. pylori. However, distribution of its MIC against H. pylori in vitro has not been regularly shown (12). In the present study, the activity of metronidazole against H. pylori exhibited a bimodal phenomenon, suggesting that clinical strains of H. pylori are either highly susceptible to metronidazole or highly resistant to this drug. Since the strains tested in this study were nonelected, the findings obtained may also reflect the natural distribution of MICs of metronidazole against H. pylori in the general population.

Five methods have been applied in the testing of H. pylori susceptibility to metronidazole. The plate dilution method, which has been considered a standard susceptibility testing method (12), is not always a practical method in a routine laboratory; e.g., it is not applicable to individual strains. Its accuracy and precision have been reported to be worse than those of the E test and the disk test (15). The microdilution broth method is not practical because of the difficulty in growing H. pylori in liquid media and the potential for contamination (7, 12, 20). The breakpoint method is very simple (11), but the results are less precise and accurate and are not satisfactory when three interpretive categories are required. The E test, which was developed in 1988 (5) and found to be as easy to perform as the disk diffusion method and to give MIC results comparable to those of the agar dilution method (6, 8, 15, 16, 26), may become a practical method. However, it is expensive, which limits its use. Therefore, the disk diffusion test is the method of choice for metronidazole susceptibility testing of H. pylori. DeCross et al. (7) reported a modified Kirby-Bauer disk diffusion method and concluded that it is practical, accurate, and clinically applicable. In the present study, a 5-μg disk diffusion test was applied to determine the metronidazole susceptibility of 121 nonelected clinical H. pylori strains. It was easy to perform, and the results obtained by this technique correlated well with those obtained by the plate dilution method. However, it was noted that 13 (10.7%) false results occurred, when the plate dilution method was used as the standard. Of the 13 discrepant results, 5 (38.5%) were obtained with strains which were susceptible or intermediate by the plate dilution method but resistant by the disk diffusion test. Also, six (46.2%) were obtained with strains which were susceptible by the plate dilution method but intermediate by the disk diffusion test. This might be due to the fact that the disk diffusion test is more likely to detect metronidazole-resistant mutant subpopulations by demonstrating the inner colonies within the zones of inhibition (14, 16). Moreover, the low levels of false susceptibility (0%) and false resistance (2.3%) confirm the suitability of the disk diffusion test.

It is generally accepted that strains of H. pylori with metronidazole MICs of more than 8 μg/ml are resistant to metronidazole (11, 13, 18, 27). However, there are no generally accepted criteria for the inhibitory zone size that differentiates susceptible and resistant strains in the disk diffusion test, although several studies have used this technique (4, 7, 9, 12, 13, 15–17, 22). Disks with different amounts of metronidazole have been used. Culture media and incubation times have also varied from laboratory to laboratory. In our experience, a 5-μg disk can give good discrimination between susceptible and resistant strains, so it was used in this study to establish an
interpretive zone diameter standard for susceptibility to metronidazole.

Three interpretive categories of susceptibility were introduced in the present study. Since it has been found that strains with metronidazole MICs between 4 and 8 μg/ml could easily develop resistance to metronidazole in vitro (24), it was recommended that strains with metronidazole MICs of less than 4 μg/ml be defined as susceptible, those with MICs between 4 and 8 μg/ml be defined as intermediate, and those with MICs of >8 μg/ml be defined as resistant. Correspondingly, strains with zone diameters of less than 20 mm should be reported as resistant, those with zones of 20 to 26 mm should be reported as intermediate, and those with zones of greater than 26 mm should be reported as susceptible. No H. pylori control strain has been identified for the disk diffusion test. Therefore, the above criteria for interpretation of results obtained by the 5-μg disk diffusion test are recommended for use pending the availability of a suitable control.

In vitro synergistic activities between metronidazole, tetacycline, and bismuth salt have been observed (20, 23, 25). In the present study, the clinical efficacy of 1 week of triple therapy including these drugs was evaluated. The eradication rates were 91.2% for susceptible strains and 52.6% for resistant strains. With the three interpretive categories used, the eradication rates for susceptible, intermediate, and resistant strains were 95.9, 62.5, and 52.6%, respectively. It has been observed that once treatment fails, emergence of metronidazole resistance in the organism is almost inevitable (3, 27). In the present study, only 4.1% of susceptible strains were not eradicated while the rate of noneradication of intermediate strains was 37.5%; all of these became metronidazole resistant. This also confirms a previous study which showed that strains with metronidazole MICs between 4 and 8 μg/ml can easily develop resistance (24).

The findings in the present study indicate that pretreatment resistance and acquired metronidazole resistance might be the only factors leading to treatment failure when good compliance is achieved, despite an over 50% eradication rate for resistant strains with triple therapy. Thus, testing of H. pylori susceptibility to metronidazole and interpretation of the results before commencing antimicrobial therapy are still important. The disk diffusion test is easy to perform and gives accurate and precise results. Three interpretive categories of susceptibility may be of benefit for clinical choice of chemotherapy in eradicating H. pylori. When the strain is susceptible to metronidazole, there is little doubt that metronidazole is the first drug to be used; when the strain is intermediate, either metronidazole should be avoided or high doses should be used; when the strain is resistant, metronidazole should be replaced by other antimicrobial agents.

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REFERENCES