JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1993, p. 1971–1974
0095-1137/93/081971-04$02.00/0

Vol. 31, No. 8

Metronidazole Susceptibility Testing for *Helicobacter pylori*: Comparison of Disk, Broth, and Agar Dilution Methods and Their Clinical Relevance

ARTHUR J. DECROSS,[1]* BARRY J. MARSHALL,[1] RICHARD W. MCCALLUM,[1] SUSIE R. HOFFMAN,[1] LEAH J. BARRETT,[2] AND RICHARD L. GUERRANT[2]

Divisions of Gastroenterology[1] and Geographic Medicine,[2] Department of Medicine, University of Virginia, Charlottesville, Virginia 22908

Received 21 January 1993/Accepted 28 April 1993

Since the methods for metronidazole susceptibility testing of *Helicobacter pylori* have not been standardized or validated, we compared three methods that are used to test the metronidazole susceptibilities of 25 isolates of *H. pylori*. Specifically, we examined the methods of Steer's replicator agar dilution, tube broth microdilution, and modified Kirby-Bauer disk diffusion. The metronidazole disk zone sizes obtained by the disk diffusion method correlated well ($r = 0.74$) with the MICs obtained by the agar dilution method. Afterward, the disk diffusion method was used to characterize the metronidazole susceptibilities of 44 isolates of *H. pylori*. Dual therapy (bismuth and metronidazole) proved to be highly effective against metronidazole-susceptible strains (81.6% eradication rate) but fared poorly against resistant strains (16.7% eradication rate; $P < 0.01$). Using agar dilution testing, we validated the modified Kirby-Bauer disk diffusion method for metronidazole susceptibility testing of *H. pylori* and conclude that it is practical, accurate, and clinically applicable.

Metronidazole resistance among *Helicobacter pylori* strains is endemic in Third World countries, and about 15 to 35% of the strains in Western countries are resistant as well (7, 18). The capacity to test metronidazole susceptibility prior to initiating therapy permits physicians to tailor therapy appropriately, in the hope of saving time, money, and patient tolerance. Testing for metronidazole susceptibility after an apparent therapeutic failure permits a more accurate evaluation of the failure. More rational second-line therapies can then be selected.

The purpose of the investigation described here was to specifically examine the utilities of several methods of testing for metronidazole susceptibility. The Steer's replicator agar dilution procedure for obtaining MICs was compared with a tube broth microdilution procedure and the modified Kirby-Bauer disk diffusion susceptibility test. Subsequently, the clinical applicability of the modified Kirby-Bauer disk diffusion method was explored by measuring the eradication rates of dual therapy (bismuth plus metronidazole) against susceptible and resistant strains of *H. pylori* as determined by that method. Our group was among the first to report on the utility of the modified Kirby-Bauer disk diffusion method in testing the metronidazole susceptibilities of *H. pylori* strains (14). This report updates our clinical experience in the application of the method.

MATERIALS AND METHODS

Preparation of bacterial suspensions. *H. pylori* isolates were grown on horse blood agar plates (150-mm-diameter plate, Columbia agar with 7% horse blood) containing GCHI enrichment (Remel, Lenexa, Kans.) for 4 to 5 days at 37°C in a 10% CO_2 incubator. Each plate was swabbed with a sterile cotton-tipped applicator, and the applicator was inoculated into *H. pylori* broth (brucella broth plus 5% fetal calf serum [we have recently switched to Trypticase soy broth for the inoculum in our disk susceptibility testing, without any apparent effect on the growth characteristics of the isolates]) to produce a cloudy suspension (McFarland no. 3 to 4). The same suspension of each isolate was used immediately for each test. Although colony counts were not performed for this experiment, our prior experience has shown that *H. pylori* is very fastidious, and a McFarland no. 3 to 4 suspension generally yields counts of only 5×10^6 CFU/ml.

Steer's replicator agar dilution procedure. The *H. pylori* suspension was divided into aliquots (200 μl), and the aliquots were placed into the wells of an aluminum seed plate. The inoculating head was lowered into the wells, raised, and then lowered onto a metronidazole-containing horse blood agar plate. This was repeated for each metronidazole concentration. Plates were allowed to dry briefly and were then incubated at 37°C in a 10% CO_2 incubator. After 5 to 7 days, the plates were examined for visible growth. The lowest concentration of antibiotic resulting in complete inhibition of growth was taken as the MIC.

Tube broth microdilution procedure. *H. pylori* broth was added to wells 2 to 12 of a 96-well sterile microtiter plate. Metronidazole was added to wells 1 and 2. An automatic microdilutor (Dynatech) was used to serially transfer from wells 2 through 11. The *H. pylori* suspension was added to the entire row of wells (e.g., rows 1 to 12); the final volume in the wells was 200 μl. The plate was incubated for 5 to 7 days at 37°C in a microaerophilic environment (85% N_2, 10% CO_2, and 5% O_2 in a Campy bag [InfoLab]) and was observed for growth (turbidity). A sterile transfer plate (Dynatech) was placed into the 96-well plate and was then transferred onto a horse blood agar plate. The agar plates were then incubated in the microaerophilic environment described above at 37°C for 5 to 7 days and then examined for growth. Turbidity in the 96-well plate was used as an indicator of the MIC, and growth on the horse blood agar plate was used as an indicator of the MBC.

Modified Kirby-Bauer disk diffusion procedure. A swab was dipped into the *H. pylori* suspension, squeezed out, and

* Corresponding author.

TABLE 1. Comparison between modified Kirby-Bauer disk diffusion method and the Steer's replicator agar dilution procedure for metronidazole susceptibility testing of *H. pylori*

Strain no.	Disk diffusion zone diam (mm)	Agar dilution MIC (μg/ml)
1	6	>32
2	6	>32
3	6	>32
4	6	16
5	6	>32
6	6	>32
7	6	4
8	9	4
9	9	2
10	11	2
11	13	1
12	14	1
13	16	4
14	18	4
15	20	0.25
16	23	2
17	25	1
18	28	1
19	30	2
20	30	8
21	30	2
22	32	0.25
23	38	2
24	40	0.5
25	40	1

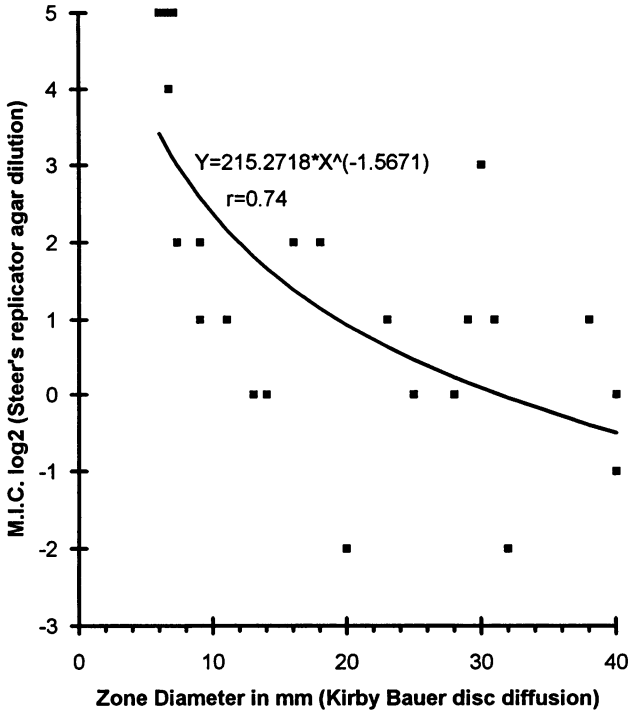

FIG. 1. Correlation between zone diameter and MIC.

streaked in three directions across a horse blood agar plate. The plates were briefly dried, and then metronidazole disks were added (6-mm disk containing 5 μg of metronidazole per ml). The plates were incubated at 37°C in a 10% CO_2 incubator for 5 to 7 days and then examined for the diameter of the zone of growth inhibition.

Multiple replications of each test for each isolate were not performed. We have previously evaluated numerous isolates for the reproducibilities of the modified Kirby-Bauer disk diffusion zone diameters and the Steer's replicator agar dilution MICs, and both tests give consistent values for a given isolate. Disk diffusion zone diameters are exactly reproducible in several replications (this has been checked for over 10 isolates). Furthermore, we have found the Steer's replicator agar dilution to give consistent MICs when the test has been repeated as many as four times for a given isolate. Seven isolates were tested repetitively in this manner by using the Steer's procedure, and results never varied by more than one (twofold) tube dilution. It should be noted that our clinical *H. pylori* isolates represent clones of a single *H. pylori* colony from each patient, because it is our practice to subculture a single colony after culturing *H. pylori* directly from a gastric biopsy specimen. This eliminates any discrepancies that might result from having more than one phenotype present in the initial *H. pylori* clinical isolate.

Clinical data. A retrospective review of our data base revealed 44 patients who had received dual therapy (Pepto-bismol; two tablets four times a day, and metronidazole, 500 mg three times a day, for 2 weeks) as their first treatment for an *H. pylori* infection. None of the 44 patients had been previously treated for *H. pylori* by any physician or had known recent exposures to either drug. A pretreatment gastric antral biopsy specimen for culture was obtained at the time of upper endoscopy. The biopsy specimen was placed in sterile saline and was immediately transferred to our laboratory, where the specimen was minced, spread onto a horse blood agar plate, and grown under microaerophilic conditions as detailed above. The clinical isolates from each patient were then individually tested for metronidazole susceptibility by the modified Kirby-Bauer disk diffusion method detailed above. We were blinded to the result of the susceptibility testing until after our posttreatment assessment of bacterial eradication, which was performed with a [^{14}C]urea breath test (15, 16) 4 to 5 weeks after the completion of therapy.

RESULTS

Twenty-five *H. pylori* strains were tested by both the modified Kirby-Bauer disk diffusion method and the Steer's replicator agar dilution procedure (Table 1). The correlation between the MIC and the zone diameters was generally good, with $r = 0.74$ (Fig. 1). The reported MIC of metronidazole for 90% of *H. pylori* tested was 1 to 4 μg/ml (15), which corresponded to a disk diffusion diameter of approximately 15 mm. The modified Kirby-Bauer disk diffusion method was far easier to perform than the Steer's replicator agar dilution procedure.

Eleven strains were tested by the tube broth microdilution procedure, but it was difficult to maintain reproducible growth in broth culture over several days with many of the isolates. Overgrowth did not occur, despite the use of a McFarland no. 3 to 4 suspension, because, as discussed above, this suspension density is necessary when working with the very fastidious *H. pylori*. The tube broth microdilution procedure was a cumbersome and time-consuming assay.

The bacterial eradication rate of dual therapy among metronidazole-susceptible isolates (zone diameter, ≥15 mm) was 81.6%, compared with a bacterial eradication rate of

16.7% ($P < 0.01$) among metronidazole-resistant isolates (zone diameter, <15 mm).

DISCUSSION

Less than 10 years from its initial isolation (17), *H. pylori* (formerly *Campylobacter pylori*) is now accepted as the major cause of active chronic gastritis (type B) (4, 5), and it has been acknowledged as a significant etiologic agent in the pathogenesis of duodenal ulcer disease (8, 21). Evidence is accumulating for the role of *H. pylori* in the natural history of gastric ulcer (9) and gastric carcinoma (19). The increasing clinical significance of this infection necessitates effective therapeutic strategies for its eradication.

Unfortunately, *H. pylori* is a relatively difficult infection to treat. The gastric habitat offers sanctuaries beneath the mucous layer and within the lumen of gastric glands and pits that partially shelter *H. pylori* from the topical or luminal effects of some antibiotics. Gastric acidity inactivates many other antibiotics. Furthermore, *H. pylori* has shown a propensity to rapidly acquire resistance to many classes of antibiotics after exposure to those agents in the form of monotherapy. These include the fluoroquinolones, the macrolides, the nitroimidazoles (including metronidazole), and rifampin (1).

For these reasons, very few antibiotic combinations are generally effective against *H. pylori*. The officially recommended first-line therapy is commonly referred to as "triple therapy," consisting of a 2-week course of bismuth, tetracycline, and metronidazole (1). This generally provides an eradication rate of between 80 and 90%. However, many patients are intolerant of the side effects of such multiple antibiotic therapy, with up to 50% of patients reporting some degree of intolerance (3). Medication intolerance will affect compliance, and it is has been shown that compliance is an important factor in the success of triple therapy (10). The presence of metronidazole-resistant *H. pylori* within the study population has also been shown to have a considerable impact upon the success of triple therapy; one recent report on 40 patients noted a 90.5% bacterial eradication rate for triple therapy (a 2-week course) against metronidazole-susceptible strains but only a 31.6% bacterial eradication rate against metronidazole-resistant strains ($P < 0.01$) (3). Metronidazole-resistant strains accounted for 48% of that study population from the United Kingdom. However, it should be acknowledged that the precise impact of metronidazole resistance upon successful triple therapy is still a controversial issue. Although others have reported a poor performance (19% bacterial eradication rate) for a 1-week course of modified triple therapy (bismuth, metronidazole, and amoxicillin) against metronidazole-resistant strains (12), this has been blamed on the duration of therapy. At least two separate investigators have claimed that a 2-week course of modified triple therapy can overcome metronidazole resistance, with bacterial eradication rates against resistant strains of 71% (6) and 63% (20), respectively. Nonetheless, this stands in contrast to the 31.6% bacterial eradication rate against metronidazole-resistant strains reported by Bell et al. (3), who used a 2-week course of therapy.

It would thus be desirable to balance the need for simpler and more easily tolerated therapies (to enhance compliance) against the metronidazole resistance of specific patient isolates. Such a balance could be struck by the use of pretreatment metronidazole susceptibility testing to appropriately guide initial therapeutic choices. Susceptible strains would be treated with a metronidazole-based therapy. We report here on the results of dual therapy (bismuth plus metronidazole) as a validation of the clinical utility of the modified Kirby-Bauer disk diffusion method, and other reports have found equivalent results for the efficacy of dual therapy (80% effective against susceptible strains, but only 15% effective against resistant strains) (22). Nonetheless, we would recommend the use of caution in the application of dual therapy against selected metronidazole-susceptible isolates of *H. pylori* when it is clear that triple therapy can achieve higher eradication rates against such selected metronidazole-susceptible isolates, as noted above. Resistant strains, on the other hand, may be treated with one of several proposed second-line therapies, such as omeprazole-amoxicillin (reported bacterial eradication rates as high as 82%) (2), omeprazole-clarithromycin (reported bacterial eradication rate of 80%) (11), or bismuth-tetracycline-erythromycin base-omeprazole (reported bacterial eradication rate of 70%) (13).

The modified Kirby-Bauer disk diffusion method of testing for metronidazole susceptibility is easy to perform, correlates well with the MICs obtained by the Steer's replicator agar dilution procedure, and is clinically applicable. We recommend its use for consideration in the selection of an optimal approach to treating *H. pylori* infections.

ACKNOWLEDGMENT

The Division of Geographic Medicine is supported in part by the Rockefeller Foundation.

REFERENCES

1. **Axon, A. T. R.** 1991. Helicobacter pylori therapy: effect on peptic ulcer disease. Part V. Working Party Report of the World Congress of Gastroenterology, 1990. J. Gastroenterol. Hepatol. **6:**131.
2. **Bayerdorffer, E., G. A. Mannes, A. Sommer, W. Hochter, J. Weingart, R. Hatz, N. Lehn, G. Ruckdeschel, P. Dirschedl, and M. Stolte.** 1992. High dose omeprazole treatment combined with amoxicillin eradicates H. pylori. Gastroenterology **102**(4, part 2):A38.
3. **Bell, G. D., K. Powell, S. M. Burridge, A. Pallecaros, P. H. Jones, P. W. Gant, G. Harrison, and J. E. Trowell.** 1992. Experience with triple anti-Helicobacter pylori eradication therapy: side effects and the importance of testing the pre-treatment bacterial isolate for metronidazole resistance. Aliment. Pharmacol. Ther. **6:**427–435.
4. **Blaser, M. J., and W. R. Brown.** 1989. Campylobacters and gastroduodenal inflammation. Adv. Intern. Med. **34:**21.
5. **Dooley, C. P., and H. Cohen.** 1988. The clinical significance of Campylobacter pylori. Ann. Intern. Med. **108:**70–79.
6. **Glupczynski, Y., and A. Burette.** 1992. Eradicating Helicobacter pylori. Lancet **339:**54–55. (Letter.)
7. **Glupczynski, Y., A. Burette, E. De Koster, J. F. Nyst, M. Deltenre, S. Cadranel, L. Bourdeaux, and D. DeVos.** 1990. Metronidazole resistance in Helicobacter pylori. Lancet **335:** 976–7. (Letter.)
8. **Graham, D. Y., G. M. Lew, D. G. Evans, D. J. Evans, and P. D. Klein.** 1991. Effect of triple therapy (antibiotics plus bismuth) on duodenal ulcer healing Ann. Intern. Med. **115:**266–269.
9. **Graham, D. Y., G. M. Lew, P. D. Klein, D. G. Evans, D. J. Evans, Z. A. Saeed, and H. M. Malaty.** 1992. Effect of treatment of Helicobacter pylori infection on the long-term recurrence of gastric or duodenal ulcer. Ann. Intern. Med. **116**(9):705–708.
10. **Graham, D. Y., G. M. Lew, H. M. Malaty, D. G. Evans, D. J. Evans, P. D. Klein, L. C. Alpert, and R. M. Genta.** 1992. Factors influencing the eradication of Helicobacter pylori with triple therapy. Gastroenterology **102:**493–496.
11. **Logan, R. P. H., P. A. Gummett, B. T. Hegarty, M. M. Walker, J. H. Baron, and J. J. Misiewicz.** 1992. Clarithromycin and omeprazole for Helicobacter pylori. Lancet **340:**239. (Letter.)
12. **Logan, R. P. H., P. A. Gummett, J. J. Misiewicz, Q. N. Karim, M. M. Walker, and J. H. Baron.** 1991. One week eradication

regimen for Helicobacter pylori. Lancet **338:**1249–1252.
13. **Marshall, B. J.** 1991. Treatment of Helicobacter pylori, p. 160–186. *In* B. J. Marshall, R. W. McCallum, and R. L. Guerrant (ed.), Helicobacter pylori in peptic ulceration and gastritis. Blackwell Scientific Publications, Cambridge, Mass.
14. **Marshall, B. J., L. J. Barret, R. W. McCallum, and R. L. Guerrant.** 1989. C. pylori therapy: is in vitro disc testing with metronidazole worthwhile? (poster). Vth International Workshop on Campylobacter Infections, Puerto Vallarta, Mexico.
15. **Marshall, B. J., M. W. Plankey, S. R. Hoffman, C. L. Boyd, K. R. Dye, H. F. Frierson, R. L. Guerrant, and R. W. McCallum.** 1991. A 20 minute breath test for Helicobacter pylori. Am. J. Gastroenterol. **86:**438–445.
16. **Marshall, B. J., and I. Surveyor.** 1988. Carbon-14 urea breath test for the diagnosis of Campylobacter pylori associated gastritis. J. Nucl. Med. **29:**11–16.
17. **Marshall, B. J., and J. R. Warren.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet **i:**1311–1315.
18. **McNulty, C. A., and J. C. Dent.** 1988. Susceptibility of clinical isolates of Campylobacter pylori to twenty one antimicrobial agents. Eur. J. Clin. Microbiol. Infect. Dis. **7:**566–569.
19. **Parsonnet, J., G. D. Friedman, D. P. Vandensteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley.** 1991. Helicobacter pylori infection and the risk of gastric carcinoma. N. Engl. J. Med. **325:**1127.
20. **Rautelin, H., T. U. Kosunen, and K. Seppala.** 1992. Eradicating Helicobacter pylori. Lancet **339:**55. (Letter.)
21. **Rauws, E. A., and G. N. Tytgat.** 1990. Cure of duodenal ulcer associated with eradication of H. pylori. Lancet **335:**1233.
22. **Weil, J., G. D. Bell, K. Powell, A. Morden, G. Harrison, P. W. Gant, P. H. Jones, and J. E. Trowell.** 1990. Helicobacter pylori infection treated with a tripotassium dicitrato bismuthate and metronidazole combination. Aliment. Pharmacol. Ther. **4:**651–657.