JOURNAL OF CLINICAL MICROBIOLOGY, May 1996, p. 1232–1234 Vol. 34, No. 5
0095-1137/96/$04.00+0

Metronidazole and Clarithromycin Resistance in *Helicobacter pylori* Determined by Measuring MICs of Antimicrobial Agents in Color Indicator Egg Yolk Agar in a Miniwell Format

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Received 22 May 1995/Returned for modification 7 September 1995/Accepted 25 January 1996

Resistance of *Helicobacter pylori* to metronidazole often causes failure of commonly used combination drug treatment regimens. We determined the MICs of metronidazole and clarithromycin against 18 *H. pylori* strains from Peru using tetrazolium egg yolk (TEY) agar. The MIC results obtained by agar dilution with petri dishes were compared with the results found through a miniwell format. The results of the two protocols for measuring drug susceptibility differed by no more than 1 dilution in all cases. On TEY agar, bright-red *H. pylori* colonies were easy to identify against a yellow background. Sixty-one percent (11 of 18) of the strains were resistant to metronidazole (MIC, ≥4 μg/ml) and 50% (9 of 18) were resistant to clarithromycin (MIC, ≥0.125 μg/ml), whereas none (0 of 5) of the strains tested were resistant to tetracycline (MIC, ≥1 μg/ml). Thus, the prevalence of metronidazole and clarithromycin resistance in Peru is higher than that in developed regions of the world. The miniwell plate with TEY agar allows easy *H. pylori* colony identification, requires about one-third less of the costly medium necessary for petri dish assaying, conserves space, and yields MICs equivalent to those with agar dilution in petri dishes.

Helicobacter pylori is associated with chronic gastritis, duodenal ulcers, and gastric cancer. It is, therefore, a major public health concern (5). While combination treatment with bismuth, metronidazole, and amoxicillin or tetracycline eradicates 80 to 90% of infections in developed countries, eradication rates in Peru are closer to 50% (4). This is likely due to the high prevalence of metronidazole-resistant strains in Peru (5). Metronidazole resistance often results in the failure of standard treatment regimens (8, 13), and, therefore, empiric therapy without knowledge of resistance patterns is suboptimal.

Determining antimicrobial resistance to *H. pylori* isolates is often difficult. *H. pylori* is fastidious and takes between 3 and 4 days for colony development. On blood agar, the colonies are small and may be hard to see on the red background. Disc testing with slowly growing bacteria is unreliable because of the long delay needed to demonstrate zone size and possible drug decay or inactivation. These technical problems are probably responsible for the relatively low correlation (0.74) between metronidazole MICs by agar dilution and by the disc test (3).

Tetrazolium egg yolk (TEY) agar was used in the present study to determine the MICs of metronidazole and clarithromycin against *H. pylori* strains from Peru. The tetrazolium salt is reduced to a colored insoluble formazan complex by growing bacteria (11, 16) and permits small colonies to be easily visualized as dark-red spots on the bright-yellow egg yolk agar. The miniwell format (with each well of a tissue culture plate used for a separate agar dilution) allows easy serial dilution of antibiotics in addition to saving incubation space and culture agar.

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MATERIALS AND METHODS

H. pylori strains were isolated from Peruvian patients with dyspepsia. None of the patients had received antimicrobial therapy during the week preceding endoscopy. Eighteen strains from 14 patients, each from a sweep of multiple colonies, were assayed for clarithromycin and metronidazole susceptibility in petri dish assays and with the miniwell format. The *H. pylori* strains were originally isolated on Skirrow's agar (brucella agar with 5% sheep blood, amphotericin [6 μg/ml], Skirrow's antibiotics [10 μg of vancomycin, 5 μg of trimethoprim, 2.5 IU of polymyxin B per ml]) and were frozen at −70°C in defibrinated sheep blood. The strains were revived and grown for 4 days on blood agar (150-mm-diameter plates) at 37°C in a microaerophilic environment (5% O_2, 85% N, 10% CO_2). Multiple colonies were picked from the plate and were suspended in phosphate-buffered saline (pH 7.4, 0.01 M) at a concentration of 1.5×10^8 to 3×10^8 CFU/ml with a McFarland no 0.5 to 1 standard. This suspension was used as the test inoculum.

Egg yolk agar was prepared as described by Westblom et al. (16), with the following modifications. The egg yolk was prepared directly, since no commercial source is readily available in Peru or in many other developing countries. The eggs were first washed with soap and water and were then immersed in 95% ethanol for 2 h at room temperature. The eggs were broken, and the yolks were separated from the albumin portions and then incorporated into the agar as described previously. Egg yolk agar contained 42.5 g of Columbia agar, 100 ml of egg yolk, and 10 ml of IsoVitaleX, to which 40 mg (filter sterilized) of 2,3,5-triphenyltetrazolium chloride (Sigma, St. Louis, Mo.) per liter was added, and the mixture was resuspended in water to a total volume of 1 liter. Immediately after the agar was poured, the plates were put in a laminar flow hood under UV lights for 2 h or were incubated for 24 h at 37°C to ensure that they were free from contamination. The plates were used within 1 week to prevent drying.

Susceptibility protocol. Isolates were assayed in two formats: a 150-mm petri dish and a 24-well tissue culture plate (Costar Corp., Cambridge, Mass.). Each well was filled with 2.0 ml of agar. The agar in both formats consisted of egg yolk

FIG. 1. Reproducibility of the MICs of metronidazole against *H. pylori* grown on miniwell TEY agar. To determine reproducibility, we used one strain in all wells. The wells in columns 1 to 4 contain, respectively, 2, 1, 0.5, and 0.25 μg of metronidazole per ml. The wells in column 5 contain agar alone, and the wells in column 6 have agar plus *H. pylori* with no antimicrobial agent.

agar as described above. Antimicrobial doubling dilutions were made and were added to the agar in final concentrations ranging from 64 to 0.125 μg/ml for clarithromycin, from 128 to 0.250 μg/ml for metronidazole, and from 64 to 0.125 μg/ml for tetracycline. The antimicrobial dilutions and IsoVitaleX were added when the egg, the Columbia agar, and the 2,3,5-triphenyltetrazolium chloride were mixed together and the mix was then poured immediately into either the wells or the petri dishes.

Seven microliters of *H. pylori* suspension containing a total of (1×10^6 to 2.1×10^6 CFU) was inoculated onto each quadrant of a 150-mm petri dish and in each well of the miniplate. For each strain, control wells or plates were filled with antibiotic-free agar. One such well or plate was then inoculated with *H. pylori* as a positive control, and another was left uninoculated as a negative control. Both the petri dishes and miniplates were incubated at 37°C in a microaerophilic environment with an anaerobic jar and a gas mixture of 5% O_2, 85% N_2, and 10% CO_2 (AGN Gas, Lima, Peru). The miniplate test used one plate, required a total of 48 ml of agar, and was able to test two strains. For the petri dish method, we used 12 plates to test each of four strains. Each plate used 15 to 20 ml of agar and was divided into four quadrants. A standard large anaerobic jar was able to contain a maximum of 25 plates for 8 susceptibility tests, while 15 miniplates were able to fit in the same jar and complete 30 susceptibility tests. All of the plates were incubated for 3 to 4 days before being read. Colonies appeared as distinct red dots on the yellow agar. All colonies were confirmed to be *H. pylori* by the urease test, which was read within 5 min. MICs were defined as the lowest concentrations of each antibiotic resulting in complete inhibition (no colonies) of growth. We considered a MIC of metronidazole of ≥4 μg/ml to be the threshold of resistance (1), and a MIC of metronidazole of greater than 32 μg/ml (15) was considered highly resistant. A MIC of clarithromycin of ≥0.125 μg/ml was considered resistant (the lower limit used in our assay), and clarithromycin MICs of >32 μg/ml were defined as highly resistant. Resistance to tetracycline was defined as a MIC of ≥1.0 μg/ml (15).

Reproducibility. Intraobserver precision was tested in the miniwell format for both metronidazole and clarithromycin. Two metronidazole-resistant and three metronidazole-susceptible strains and two clarithromycin-susceptible and two clarithromycin-resistant strains were each tested four separate times. Interobserver variation was determined by comparing the MIC results obtained by the first test of each strain with the results obtained by retesting the strains at different times as performed by different persons. Thus, two interobserver results were available for each of nine tests. Variation beyond 1 twofold dilution was considered disagreement.

Inoculum size. To determine the effect of inoculum sizes on the MICs, we tested 2 inocula of two strains for susceptibility to clarithromycin and metronidazole in the miniplate format. We tested dilutions of McFarland no. 10 and McFarland no. 0.5 to 1.0 suspensions, which correspond to 2.1×10^7 to 2.1×10^3 CFU/ml. The antimicrobial concentrations used were the same as those described above for the MIC test.

RESULTS

The results of both methods were easy to read on TEY agar, since the red colonies were strikingly visible against the yellow background. There was 100% agreement between the petri dish and miniwell formats for the MICs of both metronidazole and clarithromycin. This testing was highly reproducible with no intraobserver variation (Fig. 1). Interobserver agreement was also 100%, but three of the strains tested for metronidazole resistance varied by 1 dilution (Table 1). Variation in the inoculum size did not affect the MIC of clarithromycin but did

TABLE 1. Comparison of MICs of metronidazole, clarithromycin, and tetracycline by the miniwell versus petri dish technique[a]

H. pylori strain no.	Metronidazole MIC (μg/ml)		Clarithromycin MIC (μg/ml)		Tetracycline MIC (μg/ml)	
	Miniwell	Petri dish	Miniwell	Petri dish	Miniwell	Petri dish
1	64	64	0.25	0.25	1	1
2	1	1	0.25	0.25	<0.125	<0.125
3	2	2	<0.125	<0.125	1	1
4	4	4	<0.125	<0.125		
5	>128	>128	<0.125	<0.125		
6	0.5	1[a]	16	16		
7	4	4	>64	>64		
8	<0.25	<0.25	>64	>64		
9	128	128	2	2		
10	8	8	<0.125	<0.125		
11	1	1	2	2		
12	64	32[a]	<0.125	<0.125		
13	64	64	<0.125	<0.125		
14	4	8[a]	1	0.5[a]	<0.125	<0.125
15	0.5	0.5	<0.125	<0.125	0.25	0.5[a]
16	4	4	0.5	0.5		
17	4	4	<0.125	<0.125		
18	2	2	<0.125	<0.125		

[a] Discrepancy (1 dilution) between the miniwell and petri dish techniques. No test differed by more than 1 dilution.

slightly affect the MIC of metronidazole. Metronidazole resistance increased as the inoculum size increased. Thus, at McFarland no. 0.5 to 1.0, the MIC of metronidazole against one strain was 32 and the other was 0.5 μg/ml. When an inoculum of McFarland no. 10 was used, there was a 1-dilution increase of from 0.5 to 1.0 μg/ml in the MIC of metronidazole against one strain. The result for the other strain, for which the MIC of metronidazole was 32 μg/ml, stayed the same. For the two strains tested, the MIC of clarithromycin did not change at all dilutions at which growth occurred in the control. Inoculum concentrations of less than McFarland no. 0.5 to 1 grew more slowly and were not able to be read at 3 to 4 days.

Sixty-one percent (11 of 18) of the strains that we recovered were metronidazole resistant, and 5 of these (28% of the strains tested) were highly resistant. Although 50% (9 of 18) of our strains were clarithromycin resistant (Table 1), only 2 of 18 (11%) were highly resistant to clarithromycin.

DISCUSSION

We have shown that the miniwell technique gives results equivalent to those for the standard agar dilution technique on petri dishes; it is simple to perform and is highly reproducible. Furthermore, the dramatic color contrasts on the egg yolk agar make visualization of colonies easy and thus suitable for most clinical laboratories.

Standardization of inoculum is important, since different inoculation sizes alone can affect the estimated metronidazole MIC by as much as 16 times (1). In our study, this effect was noted to a lesser degree for metronidazole but was not observed in relationship to clarithromycin. An inoculum of McFarland no. 0.5 to 1.0 (1×10^6 to 2.1×10^6) appears to be satisfactory. Lower concentrations often do not grow adequately. In addition, a McFarland no. 0.5 is used as a standard inoculum for the E test for *H. pylori* (12). Inoculum size had little effect on the MIC of clarithromycin.

Resistance of *H. pylori* isolates to antimicrobial agents such as metronidazole is high in third world countries, where pharmacy practices are poorly regulated and overprescription of antimicrobial drugs is common. In Peru, erythromycin, clarithromycin, and metronidazole are frequently sold over the counter, although exact figures are not available (4a). Frequent exposure to macrolides and nitroimidazoles in this population is the most likely explanation for the increased prevalence of resistance to these antimicrobial agents (7). However, tetracycline is commonly used in third world countries, yet none of the five *H. pylori* strains tested were resistant to this drug.

The present study points out the high prevalence of *H. pylori* metronidazole resistance in *H. pylori* isolates from Peruvian patients, which likely accounts for the low eradication rates achieved by metronidazole-containing regimens here and in other third world sites. In addition, clarithromycin and omeprazole have been recommended for treatment of *H. pylori*. In developing countries like Peru, further studies determining the efficacy of this combination are needed, since some strains of *H. pylori* may be highly resistant to clarithromycin. Since treatment of *H. pylori* is almost never urgent, treatment should probably be delayed until metronidazole or clarithromycin susceptibility is known. When resistance is found, a regimen can be chosen that does not contain this drug, such as (i) amoxicillin and omeprazole or (ii) bismuth, furazolidone, and amoxicillin. In addition, in developed countries, a combination of triple therapy and omeprezole has been used successfully to treat metronidazole-resistant strains (2). Resistance to clarithromycin was common; however, in contrast to metronidazole, the level of clarithromycin resistance was usually low. However, the low pH of the stomach markedly affects the activity of clarithromycin; thus, even low-level resistance may be important clinically (6).

H. pylori is linked to gastric cancer (9, 10), and *H. pylori* eradication reduces long-term recurrence of gastritis and duodenal ulcers (14); therefore, appropriate therapy guided by antibiotic susceptibilities is warranted. Use of a TEY MIC assay that is performed either in miniwells or on standard plates will assist clinicians in providing appropriate therapy for this important pathogen.

ACKNOWLEDGMENTS

We thank the Japanese Peruvian Polyclinic and J. B. Phu and D. Sarah for their assistance. We also appreciate the help of K. Tanaka and R. Hallas of Abbot Co. and the comments of C. Sterling, R. Black, and R. Oberhelman.

Funding was provided in part by Abbott Labs, Merck Sharp & Dohme, the RG-ER fund, and CONCYTEC of Peru.

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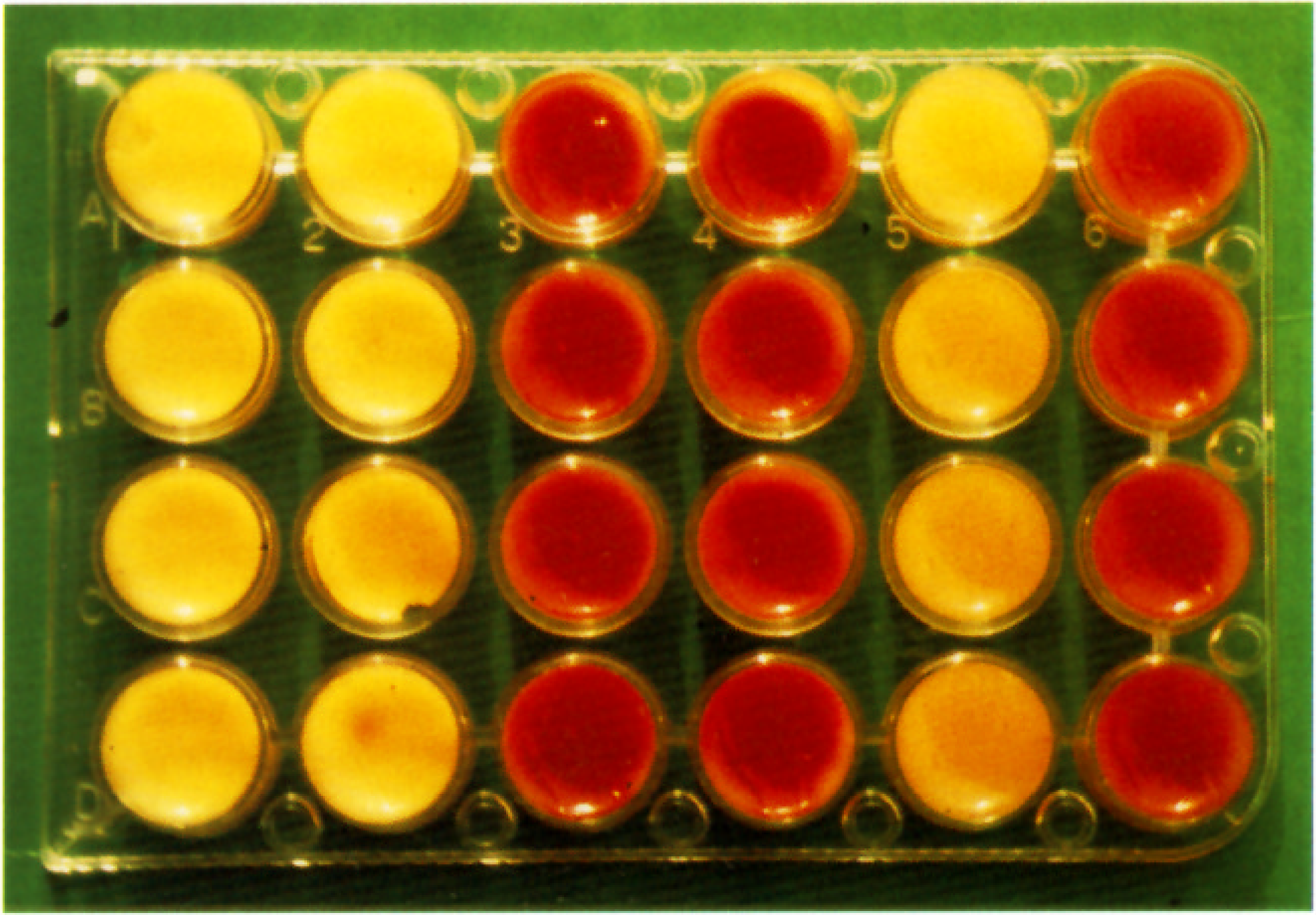
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