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Evaluation of the E Test for Quantitative Antimicrobial Susceptibility Testing of *Helicobacter pylori*

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The Progressive Diagnostics Manufacturers epsilometer test (E test; AB Biodisk, Solna, Sweden), a quantitative variant of the disk diffusion technique, was evaluated comparatively to an agar dilution method for the antimicrobial susceptibility testing of *Helicobacter pylori*. A collection of 79 *H. pylori* clinical strains, including isolates with known resistance to various antimicrobial agents, was tested against 12 different antimicrobial agents. All strains were tested on Columbia agar supplemented with 10% horse blood. Plates were incubated at 37°C in microaerobic atmo-sphere (5% O_2, 10% CO_2), and readings were done after 3 days of incubation. In general, E test MICs were easy to interpret and the correlation between MICs by the agar dilution method and the E test was good, with 86 and 99.5% of results being within, respectively, 1 and 2 $\log_2$ dilution steps in a total of 936 tests. All strains of *H. pylori* with documented resistance to the tested agents were detected by the E test. Thus, the E test appears to be an easy and reliable method for determination of MICs of antibiotics for *H. pylori*, and it may offer an interesting alternative to MIC determination by the agar dilution technique.

The Progressive Diagnostics Manufacturers (PDM) epsilometer test (E test; AB Biodisk, Solna, Sweden) is a new in vitro susceptibility testing method designed for quantitative determination of susceptibility to antimicrobial agents. The E test is a plastic strip containing a predefined, continuous, and exponential antibiotic gradient on one side and a graded continuous MIC scale that covers 15 twofold dilutions on the opposite site. To determine an MIC with the E test, the surface of an agar plate is swab inoculated with an adjusted bacterial suspension in the same manner as for a disk diffusion test. After appropriate incubation time, the interaction of the antimicrobial agent gradient and the tested bacterial inoculum results in an elliptic inhibitory zone which, by use of the MIC scale on the strip, indicates the MIC of the drug for the organism.

It is now universally accepted that *Helicobacter pylori* is the major etiological agent of chronic type B gastritis (5, 7, 13) and that it may also be involved in the pathogenesis of peptic ulcer disease (12, 17). The treatment of *H. pylori* infection is difficult, and it appears from the initial clinical trials that in vitro activity does not always correlate with in vitro success and that relapse frequently occurs after apparently successful elimination of the organism with various antimicrobial agents and/or bismuth salts. Increasing resistance of *H. pylori* to several classes of antimicrobial agents has further complicated the search for an optimal treatment regimen (6) and has also focused new attention towards reliable methods for determining in vitro susceptibility of this bacterial species.

In fact, there are currently no standard methods nor any optimal procedures for testing the susceptibility of *H. pylori* to antibiotics, and most investigators have used either the disk diffusion test or the MIC agar dilution technique, because these procedures are usually applied in the microbiology laboratory for the testing of other microorganisms (15, 16).

The purpose of this study was to evaluate the E test for determining quantitative susceptibility of *H. pylori* to antimicrobial agents of possible clinical relevance for this species.

E test strips containing ampicillin, aztreonam, cefaclor, cefuroxime, ciprofloxacin, clindamycin, erythromycin, gentamicin, metronidazole, nitrofurantoin, oxacillin, and tetracycline were purchased from AB Biodisk (Solna, Sweden) for the purpose of this study. Reagent-grade powders of the same antimicrobial agents were used for agar dilution MIC tests. All antimicrobial solutions were prepared within 2 days of use according to the procedure of the manufacturer. Sixty consecutive nonduplicate clinical strains of *H. pylori* isolated from gastric antral biopsies and identified by accepted criteria (7) were used. In addition, a collection of 19 selected strains of *H. pylori* with various resistance patterns and levels of antimicrobial susceptibility was also utilized for this evaluation: 10 strains resistant to metronidazole (MIC, >32 μg/ml), 4 resistant to erythromycin (MIC, >64 μg/ml) and clindamycin (MIC, >64 μg/ml), 4 resistant to ciprofloxacin (MIC, ≥4 μg/ml), and 1 resistant both to metronidazole (MIC, >32 μg/ml) and to ciprofloxacin (MIC, 4 μg/ml). These latter strains were isolated from patients who had been enrolled in several treatment trial protocols and for whom MICs for the infecting strain had been previously determined by an agar dilution method (6). Control strains employed in this study included *H. pylori* NCTC 11637 and NCTC 11638, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923.

E test MICs for *H. pylori* were performed on Columbia agar (B-D Microbiology Systems, Cockeysville, Md.) supplemented with 10% horse blood. Inocula were prepared from two agar plates of a 2-day fresh growth on Columbia blood agar which were scraped and suspended in 5 ml of Columbia broth to achieve a turbidity equivalent to a McFarland opacity standard of 3 to 4. Serial dilutions of this

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TABLE 1. Comparative E test MICs of 12 antimicrobial agents for unselected consecutive strains of *H. pylori*

Antibiotic	MIC[a] (μg/ml)		
	Range	50%	90%
Ampicillin	<0.016–0.06	<0.016	0.016
Aztreonam	<0.047–1	0.38	0.75
Cefaclor	<0.016–2	0.047	1
Cefuroxime	<0.016–0.5	0.023	0.38
Ciprofloxacin	0.004–0.094	0.032	0.094
Clindamycin	0.064–2	0.5	2
Erythromycin	<0.016–0.25	0.064	0.19
Gentamicin	0.25–2	0.5	1
Metronidazole	0.064–>32	1	>32
Nitrofurantoin	0.064–12	0.25	0.5
Oxacillin	0.25–8	1	4
Tetrracycline	<0.016–0.19	0.047	0.125

[a] 50% and 90%, MIC for 50 and 90% of isolates, respectively.

bacterial suspension were made for both *H. pylori* National Collection of Type Cultures control strains and yielded colony counts of about 0.5×10^9 CFU/ml. Screening of the influence of the inoculum size on the antibacterial activity was made against four selected strains, including one reference strain (*H. pylori* NCTC 11637), by using 10-fold dilutions ranging between 0.5×10^6 and 0.5×10^9 CFU/ml. In addition, the shape and motility of the organisms were controlled before inoculation by gram stain and phase-contrast microscopy. Cultures showing a high proportion (about 25% or more) of rounded, nonmotile bacterial forms were discarded. The 140-mm-diameter agar plates were inoculated by confluent swabbing of the surface with the adjusted inoculum suspensions. Inoculated plates were allowed to dry before E test strips were applied to the medium. After application of the E test strips (with a maximum of four strips per agar plate), plates were incubated at 37°C under a microaerophilic atmosphere (5% O_2, 10% CO_2, 85% N_2) (Incubator Forma Scientific, Ann Arbor, Mich.) for 72 h. E test results were interpreted by recording the point of intersection of the growth elliptic margin zone with the MIC scale value on the E test strip. Agar dilution MIC tests were performed by using twofold concentrations increments of the antimicrobial agents incorporated in molten Columbia blood agar in the manner suggested by the National Committee for Clinical Laboratory Standards standard M7-A2 (15). Inoculum suspensions were prepared as described above and then diluted in Columbia broth and delivered to the surface of the agar plates with a Steers replicator apparatus, which resulted in a final inoculum of ca. 5×10^5 CFU per spot.

Agar dilution plates were incubated for 72 h at 37°C under similar microaerophilic conditions before interpretation of the MICs in the usual manner (15).

The results of the E test MICs of the sixty consecutive and nonselected *H. pylori* strains are shown in Table 1. Overall, these results are in accordance with those already obtained in various other studies (8, 11, 14). Fifteen of 60 (25%) strains were found resistant to metronidazole, thus confirming the rather high frequency with which resistance of *H. pylori* to this antibiotic occurs in the clinic (6, 9). In most cases, results were easily interpreted for most drugs, since the inhibition ellipses were generally clearly demarcated and the point of intersection of the zone edge with the strip was also well delineated. Reading of the E tests was also without problem for the vast majority of the selected *H. pylori* strains displaying resistance to one or more antimicrobial agents. However, for metronidazole, some strains yielded growth of numerous small colonies within the inhibition ellipse zone. These colonies were usually clearly identified and were interpreted as resistant isolates. A few strains yielded poorly defined diffuse elliptic inhibition zones, especially with aztreonam, erythromycin, and tetracycline. On repeating these tests, the same phenomenon was usually seen and reproducible, and the MIC result interpretation did not change. In addition, the MIC results of the various antibiotics were always identical or within 1 $\log_2$ dilution step for the four strains that were tested with different inocula ranging between 0.5×10^6 and 0.5×10^9 CFU/ml.

The correlation between the results of tests by agar dilution and E test methods for the 12 agents is shown in

TABLE 2. Comparison of *H. pylori* E test MICs and agar dilution MICs in 936 tests

Drug (no. of strains[a])	No. of E test MICs (%[a]) within indicated number of $\log_2$ dilution steps of agar dilution MICs							Results out of range[b]	
	>−2	−2	−1	0	+1	+2	>+2	One	Both
Ampicillin (47)	0	2 (4.2)	13 (27.7)	24 (51.1)	8 (17.0)	0	0	8	24
Aztreonam (79)	1 (1.3)	3 (3.8)	24 (30.4)	35 (44.3)	16 (20.6)	0	0	0	0
Cefaclor (76)	0	4 (5.3)	17 (22.4)	43 (56.6)	12 (15.8)	0	0	3	0
Cefuroxime (70)	0	8 (11.4)	28 (40.0)	7 (10.0)	26 (37.1)	1 (1.4)	0	5	3
Ciprofloxacin (77)	0	14 (18.2)	54 70.1)	9 (11.7)	0	0	0	2	0
Clindamycin (75)	0	3 (4.0)	24 (32.0)	20 (26.7)	23 (30.7)	5 (6.7)	0	0	4
Erythromycin (73)	0	0	21 (28.8)	32 (43.8)	16 (21.9)	4 (5.5)	0	2	4
Gentamicin (79)	0	1 (1.3)	12 (15.2)	47 (59.5)	14 (17.7)	5 (6.3)	0	0	0
Metronidazole (68)	0	5 (7.4)	18 (26.5)	28 (41.2)	11 (16.2)	6 (8.8)	0	9	2
Nitrofurantoin (73)	0	4 (5.5)	28 (38.4)	26 (35.6)	13 (17.8)	1 (1.4)	1 (1.4)	0	0
Oxacillin (72)	2 (2.8)	23 (31.9)	39 (54.2)	6 (8.3)	2 (2.8)	0	0	2	0
Tetracycline (70)	1 (1.4)	27 (38.6)	40 (57.1)	2 (2.9)	0	0	0	7	2
Total (859)	4 (0.5)	94 (10.9)	318 (37.0)	279 (32.5)	141 (16.5)	22 (2.6)	1 (0.1)	38	39

[a] Correlation of strains with MICs within the concentration range of the E test. The total number of strains tested against each drug equals the in-range and out-of-range results for each drug.

[b] Either one or both of the agar dilution MIC and E test results were outside the concentration range of the E test.

Table 2. Overall, 86% of results were within 1 $\log_2$ dilution step and 99.5% were within 2 $\log_2$ dilution steps. With all individual antimicrobial agents, most results by the two methods were within 1 $\log_2$ dilution step and results rarely differed by more than 2 $\log_2$ dilution steps (Table 2). However, with ciprofloxacin, oxacillin, and tetracycline the results were frequently 1 or 2 $\log_2$ dilution steps lower by the E test, while there was no particular trend for MICs of the other antibiotics compared with the agar dilution MICs. With ampicillin, a comparison between the two methods was, however, possible in only 47 of 79 (59.5%) of the strains, since with the remaining strains one or both MIC results were outside the lowest drug concentration. Correlation between the two methods was also found to be excellent (100% within ±1 dilution step) against the 19 selected antimicrobial resistant strains, and no major or very major errors were found between E test and agar dilution MICs of erythromycin, clindamycin, ciprofloxacin, and metronidazole. However, a quantitative comparison could not be made against these antimicrobial resistant isolates, since one or both MIC results were systematically outside the range of concentration of the drugs.

Determination of the susceptibility of *H. pylori* to antimicrobial agents may be of growing importance, since it appears that primary or acquired resistance to various drugs may be responsible for failure to eradicate this bacterium from the stomach. Presently, there are no standard methods for the determination of in vitro susceptibility of this fastidious organism. Furthermore, besides the lack of methodological recommendations for testing the susceptibility of *H. pylori* to antimicrobials, the cutoff levels of resistance to drugs have not been determined. Criteria for defining resistance to a particular drug should be based on local gastric concentration and not on levels in serum, as is usually done with other microorganisms. Since determination of stomach drug level may be considered too tedious a task, one approach to circumventing this problem would be to determine the susceptibility of *H. pylori* to antimicrobial agents against clinical isolates both before and after treatment and to infer the cutoff level for resistance on the basis of MICs for posttreatment isolates when development of resistance does occur.

The E test represents a new and innovative approach to the quantitative determination of antimicrobial susceptibility which is potentially applicable to a wide range of drugs and microorganisms. Two separate studies have shown that the E test results were as reliable as the results obtained by broth microdilution and agar dilution tests, with the overall agreement between the E test and the standard susceptibility testing methods being equal to or greater than 95% (1, 3). In particular, the E test approach might be interesting for testing fastidious bacteria (10) or bacteria that are difficult to test (e.g., anaerobes [2]). This test is technically very simple and needs no special equipment, and the methodology is familiar to most laboratories, since tests are performed in a manner that is very similar to the agar disk diffusion method. The stability of the antimicrobial gradient produced by the E test limits the effects of the bacterial inoculum size, preincubation, and prediffusion, which generally have a marked influence on the results of disk diffusion tests (4). The versatility and ease of use of the E test make the method of considerable appeal in situations in which only a few organisms or a few drugs need to be tested, a situation which is likely to be encountered with difficult organisms such as *H. pylori*.

The present study has demonstrated the potential use of the E test for determination of the susceptibility of *H. pylori* to various drugs. A correlation between MICs by the agar dilution and E test methods was good in that almost 100% of the results were within 2 $\log_2$ dilution steps, and no major discrepancies between these methods upon testing of organisms with known patterns of resistance to several drugs were observed. Except for a slight tendency by the E test towards lower MIC values of ciprofloxacin, oxacillin, and tetracycline, no significantly discordant results were found with any particular antibiotics, indicating that the E test method may apply for testing the susceptibility of *H. pylori* to a wide array of drugs.

However, because of the exquisite susceptibility of *H. pylori* strains to most antimicrobial agents, no more than four E test strips should be placed on the surface of a 140-mm-diameter round petri dish in order to avoid overlaps between the inhibition zone sizes which would affect the reading of results. Considering the cost of this method (about U.S. $2.50 per test, in Belgium) it is probable that this method will not prove to be very economical, especially for testing a large number of drugs on numerous isolates. It could, however, be worth considering for occasional testing of fastidious bacteria (such as *H. pylori*) against selected drugs.

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