Comparison of activity of 10 antibiotics against clinical strains of *Helicobacter pylori* by three different techniques

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K Weiss, M Laverdière, C Restieri. Comparison of activity of 10 antibiotics against clinical strains of *Helicobacter pylori* by three different techniques. Can J Gastroenterol 1998;12(3): 181-185. The authors determined the susceptibility of 55 single clinical strains of *Helicobacter pylori* isolated in the Montreal area to 10 antibiotics by three different methods – an agar dilution technique considered to be the gold standard, a disk diffusion method and the E-test. Testing was performed on Mueller-Hinton agar supplemented with 10% sheep blood; plates were incubated at 37°C for 72 h in a microaerophilic atmosphere. The metronidazole resistance rate is about 11% in the Montreal area. Macrolides are very active against *H pylori* isolates, with few variations in activity between older and newer molecules. Correlation among different methods was not as good as reported in the literature for metronidazole.

Key Words: *Agar dilution, Disk diffusion, E-test,* Helicobacter pylori

Comparaison de l'activité de 10 antibiotiques contre des souches cliniques de *Helicobacter pylori* au moyen de trois techniques différentes

RÉSUMÉ : Les auteurs ont déterminé la susceptibilité de 55 souches cliniques uniques de *Helicobacter pylori* isolées dans la région de Montréal à 10 antibiotiques, au moyen de trois méthodes différentes qui sont la technique par dilution en gélose, considérée comme la technique de référence, la méthode par diffusion des disques et le test E. On a pratiqué les tests sur de la gélose de Mueller-Hinton à laquelle on a ajouté 10 % de sang de mouton. Les boîtes de Pétri ont été placées dans un incubateur à 37°C pendant 72 heures dans un milieu micro-aérophile. Le taux de résistance au métronidazole est d'environ 11 % dans la région de Montréal. Les macrolides sont très actifs contre les isolats de *H. Pylori*, avec peu de variations dans l'activité entre les molécules plus anciennes et les nouvelles molécules. La corrélation entre les différentes méthodes pour le métronidazole ne s'est pas révélée aussi bonne que celle rapportée dans la littérature.

Helicobacter pylori is one of the most common infections in terms of number of people infected (up to 50% of the world's population [1]), and the bacterium has been recently designated a definite carcinogen by the World Health Organization. This implies that antibiotic use has to be controlled to avoid potentially serious resistance problems.

Treatment of medical conditions associated with the presence of *H pylori* consists mainly of oral antibiotics, frequently administered with a proton pump inhibitor. Treatment regimens are numerous, and efficacy may be quite variable depending on the combinations used and their duration. Nevertheless, antibiotics play a key role in curing symptomatic patients harbouring the bacterium. It is therefore essential to monitor the pattern of susceptibility of *H pylori* to antimicrobial agents.

Many parts of the world have high resistance rates to metronidazole, ranging from 6% in developed countries to 84% in third world countries (2,3), because of its extensive use for parasitic infections. Furthermore, metronidazole resistance has been associated with treatment failure in certain studies (4,5).

In Canada, *H pylori* resistance patterns to antibiotics have

This study was presented in part at the annual meeting of the Canadian Association of Gastroenterology, Quebec City, Quebec, February 23 to March 1, 1997

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Received for publication November 12, 1997. Accepted January 20, 1998

TABLE 1
Minimum inhibitory concentrations (MICs) of 55 clinical isolates of *Helicobacter pylori* to 10 antibiotics by agar dilution

Antibiotic	Range (mg/L)	MIC_{50}	MIC_{90}
Ampicillin	≤0.015-0.25	0.03	0.06
Cefuroxime	≤0.06-0.25	≤0.06	0.25
Ciprofloxacin	≤0.06-0.25	0.125	0.25
Erythromycin	≤0.06-4	≤0.06	0.125
Azithromycin	≤0.06-0.5	≤0.06	0.125
Clarithromycin	≤0.06-0.5	≤0.06	≤0.06
Roxithromycin	≤0.06-0.125	≤0.06	≤0.06
Metronidazole	0.25 - >64	2.0	16
Tetracycline	≤0.03-0.5	0.125	0.25
Clindamycin	0.125-8	0.125	4.0

Data compiled from reference 10. MIC_{50} MIC of 50% of isolates; MIC_{90} MIC of 90% of isolates

rarely been evaluated (6). One study conducted in Montreal found the rate of metronidazole resistance to be 12% (7). Macrolide resistance has been reported to be very low, with an estimate of 1.8% for clarithromycin (8).

Because metronidazole and macrolides represent the cornerstone of antibiotic therapy, it is worthwhile to determine the susceptibility rates of these agents towards *H pylori* in our community. Besides, there are no clear and established methods for determining susceptibility patterns of *H pylori*. Because *H pylori* is rather fastidious, comparing different susceptibility techniques might be helpful if there were a simple, easily applicable method for clinical diagnostic laboratories.

MATERIALS AND METHODS

Bacteria: Fifty-five single clinical strains of *H pylori* were tested against 10 antibiotics. Forty-five strains were obtained from gastroduodenal biopsies taken in the authors' endoscopy unit, and 10 strains were kindly provided by the Quebec Provincial Laboratory.

Antibiotics: The following antibiotic powders were used: ampicillin, cefuroxime, erythromycin, metronidazole, tetracycline and clindamycin (Nucrotechnics); and ciprofloxacin (Bayer Inc), azithromycin (Pfizer Canada), clarithromycin (Abbott Canada) and roxithromycin (Rhône-Poulenc Rorer). E-test strips were purchased from Oxoid Inc. The following disks were used: ampicillin (10 µg), cefuroxime (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), tetracycline (30 µg) and clindamycin (2 µg) (Remel, Kansas); clarithromycin (15 µg), roxithromycin (15 µg) and metronidazole (5 µg) (Oxoid Inc); and azithromycin (15 µg) (BBL, Maryland).

Susceptibility testing: Susceptibility was tested by three different techniques – an agar dilution considered to be the gold standard, disk diffusion and E-test (Oxoid Inc).

Inocula were prepared by suspending the bacterium in Mueller-Hinton broth in order to reach a MacFarland 5 standard, yielding a theoretical viable bacterial count of about 1.5×10^9 colony forming units (CFU)/mL (9). This suspension density is necessary because *H pylori* is very fastidious and slow growing. Suspended *H pylori* strains were then plated with a swab directly onto 140 mm diameter Mueller-Hinton agar plates (BBL) supplemented with 10% sheep blood for the disk diffusion and E-test methods. Large plates were needed because of the extensive inhibition zones recorded around antibiotic disks and E-test strips. A maximum of four E-test strips or disks were placed on each plate. For agar dilution, a multipoint inoculator dispensing 30 µL per inoculum spot of the initial suspension was plated on Mueller-Hinton plates supplemented with 10% sheep blood, reaching a theoretical final concentration of approximately 45×10^6 CFUs per spot. No specific CFU count was performed. Serial twofold dilutions of the following antibiotics were used for agar dilution: ampicillin (0.015 to 8 mg/L), cefuroxime (0.06 to 32 mg/L), ciprofloxacin (0.06

TABLE 2
Comparison of *Helicobacter pylori* agar dilution minimum inhibitory concentrations (MICs) with E-test MICs in 550 tests

	Number of E-test MICs within indicated $\log_2$ dilution compared with agar dilution MICs								
	≤–4	–3	–2	–1	0	+1	+2	+3	≥4
Ampicillin	2	0	7	15	25	6	0	0	0
Cefuroxime	5	3	2	2	36	6	0	0	0
Ciprofloxacin	3	3	11	13	16	8	1	0	0
Erythromycin	1	0	1	1	25	17	7	2	1
Azithromycin	0	4	1	2	33	9	3	2	1
Clarithromycin	0	0	0	28	21	3	3	0	0
Roxithromycin	0	0	0	15	17	16	3	1	3
Metronidazole	8	7	7	9	10	6	3	1	4
Tetracycline	9	14	13	8	7	4	0	0	0
Clindamycin	0	2	6	9	7	8	9	5	9
Total (all agents)	27	33	48	102	197	83	29	11	18
%	4.9	6	87	18.5	35.8	15.1	5.3	2	3.3

Data compiled from references 11 and 12

TABLE 3
Comparison of three different methods of testing susceptibility of *Helicobacter pylori* to 10 antibiotics. Agar dilution is the gold standard

	E-test				Disk diffusion			
	% Agreement	Very major error	Major error	Minor error	% Agreement	Very major error	Major error	Minor error
Ampicillin	100	0	0	0	100	0	0	0
Cefuroxime	100	0	0	0	100	0	0	0
Ciprofloxacin	98.2	1	0	0	98.2	0	1	0
Erythromycin	98.2	0	1	0	98.2	0	1	0
Azithromycin	98.2	0	1	0	98.2	0	1	0
Clarithromycin	100	0	0	0	100	0	0	0
Roxithromycin	98.2	0	1	0	98.2	0	1	0
Metronidazole	81.9	3	5	1	NA	NA	NA	NA
Tetracycline	100	0	0	0	100	0	0	0
Clindamycin	47.3	1	3	25	40	0	20	13

National Committee for Clinical Laboratory Standards values were used as guidelines. NA Not available

to 32 mg/L), erythromycin (0.06 to 32 mg/L), azithromycin (0.06 to 32 mg/L), clarithromycin (0.06 to 32 mg/L), roxithromycin (0.06 to 32 mg/L), metronidazole (0.125 to 64 mg/L), tetracycline (0.03 to 16 mg/L) and clindamycin (0.06 to 32 mg/L). All plates were subsequently incubated at 37°C for 72 h in a microaerophilic atmosphere, obtained by adding a gas-generating pack (campylobacter microaerophilic system, Difco) in a 2.5 L sealed jar.

Due to the slow growing nature of the microorganism, readings were recorded after 72 h of incubation for all three methods using the National Committee for Clinical Laboratory Standards (NCCLS) criteria only as general guidelines because there are no official recommendations for *H pylori* susceptibility testing (10-12) (Tables 1,2). One author suggested that the minimum inhibitory concentration (MIC) susceptibility breakpoint for metronidazole is 8 µg/mL or less (3). This value was used to determine the resistance rate in the Montreal area.

Quality control was ensured by testing *Enterococcus faecalis* American Type Culture Collection (ATCC) 29212, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213 and *Helicobacter pylori* ATCC 43504 simultaneously.

Definitions: Discrepancies were classified as minor, major or very major. The agar dilution technique was used as the gold standard; values in NCCLS tables were used to categorize the tested strains.

A minor error was defined as identification of a strain as susceptible or resistant by the disk diffusion technique or the E-test, but intermediate by agar dilution. A strain categorized as intermediate by the disk diffusion or the E-test and as either susceptible or resistant by agar dilution was also included in this definition. A major error was defined as identification of a strain as resistant by either the E-test or the disk diffusion methods, but susceptible by the agar dilution method. A very major error was defined as categorization of a strain as susceptible by either the E-test or the disk diffusion methods, but resistant by the agar dilution method. Clinical information was not available for all patients; therefore, no clinical correlation with resistance rate was determined in this study.

RESULTS AND DISCUSSION

All macrolides tested are very active against strains of *H pylori* in our community (Table 1). The MIC of 90% of isolates (MIC_{90}) was always inferior or equal to 0.125 mg/L for erythromycin, azithromycin, roxithromycin and clarithromycin. By using the available NCCLS criteria for aerobic bacteria, all the tested strains were considered susceptible. For macrolides, strains with MIC 8 mg/L or less are considered resistant. Some authors have suggested a lower level (2 mg/L) (8). *H pylori* strains are known to have a bimodal distribution of MICs – some are highly resistant, while others are highly susceptible (13). In our study, there were no major differences in activity among erythromycin, azithromycin, clarithromycin and roxithromycin. From the MIC_{90}, we determined that the latter two antibiotics were a little more active (0.06 mg/L or less versus 0.125 mg/L). The clinical significance of the minor in vitro differences in MIC_{90} among the macrolides is unknown. Although there was limited comparison of agar dilution with other methods due to the lack of macrolide-resistant strains, we did not record any very major errors with any technique (Table 3). Published data on the accuracy of the E-test or the disk diffusion method for the newer macrolides are not available. The E-test performed well in one study that tested the resistance of *H pylori* to erythromycin – only 5.5% of results were two or more $\log_2$ dilution steps from agar dilution MICs (14). In the present study, the rate was 12.6% when all macrolides tested were taken into account. The rate ranged from 5.4% for clarithromycin to 21% for erythromycin. In general, variations within a range of plus or minus two $\log_2$ dilution steps between two techniques are considered acceptable.

Macrolide resistance to *H pylori* is still low throughout the

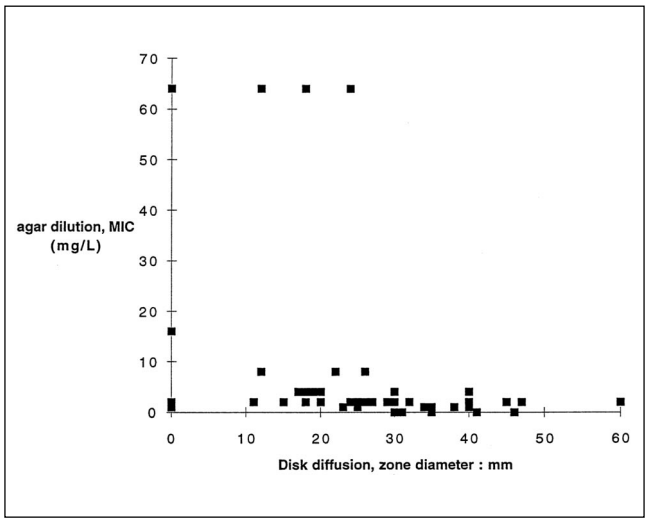

Figure 1) *Correlation of agar dilution and disk diffusion for metronidazole. MIC Minimum inhibitory concentration*

western world, but resistance rates of up to 10% have been recorded in France and Spain (13). However, in one study 50% (nine of 18) of strains originating from Peru were resistant to clarithromycin (15). In Canada, a recent study from Nova Scotia found a resistance rate of 1.8% for clarithromycin (8). This suggests that clarithromycin, a key component of many therapeutic regimens, can still be used in Canada. The mechanism of resistance to macrolides involves a lack of binding to ribosomes due to a point mutation within a conserved loop of 23S ribosomal RNA (16). Moreover, if a strain is known to be resistant to one macrolide it should be considered resistant to all others (13). Because compliance is a key factor in treatment success, newer macrolides with a long half-life that demonstrate excellent activity in vitro may be useful.

Tetracycline, which is frequently included in proposed *H pylori* treatment regimens, had excellent activity against all clinical isolates in vitro, even against strains resistant to both metronidazole and macrolides. The same pattern was noted in Peruvian strains, which have always been susceptible to tetracycline (15). This seems quite odd because tetracycline has been used considerably worldwide.

When agar dilution is considered the gold standard and a suggested breakpoint of 8 µg/mL is used (3), the resistance rate of *H pylori* to metronidazole in the authors' area is about 11%. Compared with frequencies found in other countries, this is quite low and probably reflects the relative low usage of metronidazole in Canada. This resistance rate is consistent with that found by another Montreal group (7).

Metronidazole is by far the most extensively studied antibiotic in terms of in vitro susceptibility testing against *Helicobacter pylori* (2,3,5,7,15,17). In the present study, the E-test was not a very reliable method for determining *H pylori* susceptibility to metronidazole because three very major errors were recorded (Table 3) and 36.6% of all results were three or more $\log_2$ dilution steps from agar dilution MICs (Table 2). The observed discrepancies are rather problematic because metronidazole constitutes a major component of several therapeutic regimens, and resistance to metronidazole has been associated with treatment failure (4). However, once again, the E-test method was adequate for testing other antibiotics. Similar findings have been reported by others who found the E-test to be an acceptable technique for measuring susceptibility (14,17,18). One study evaluated the E-test specifically for metronidazole and found this technique to be very acceptable, even for routine testing (17).

Disk diffusion has also been compared with other methods, and some authors have tried to determine the specific zone diameter for metronidazole. Susceptibility was considered to be a zone diameter 12 mm or greater with a 5 µg disk (3). Others defined susceptibility as an inhibition zone of 26 mm or more (5). Yet in the present study, correlation between agar dilution and disk diffusion was not optimal overall (Figure 1). A wide range of zone diameters has been found by different investigators, indicating that metronidazole susceptibility testing is a challenge; several key factors such as inoculum size (19), supporting medium and incubation time render comparison between studies difficult. The resistance mechanism of metronidazole in *H pylori* is not yet completely characterized, but anaerobic reduction seems to be blocked, resulting in futile cycling (20).

We recommend great caution in implementing other susceptibility methods for metronidazole in clinically oriented laboratories, even if they are simpler to apply than agar dilution.

Despite its extensive use, ampicillin is still very active against *H pylori* isolates from our area; no resistant strain was detected. This is particularly relevant because it implies that amoxicillin can still be widely used for treatment purposes.

H pylori susceptibility testing is nonstandardized and cumbersome and should, therefore, be restricted to clinical situations where treatment failure of adequate antibiotic regimens is strongly suspected or be used only for research purposes.

As long as there are no official recommendations, agar dilution should be used as the standard method in order to avoid potential errors (13), in particular for metronidazole. Inoculum size, appropriate testing medium, pH conditions of the medium (especially for macrolides) (21) and incubation time are all variables that must be standardized before using alternative testing methods for *H pylori* (22), particularly when assessing the activity of metronidazole (13). For macrolides, the E-test seems to be adequate, although some questions remain unanswered for this class of antibiotics (21).

All the technical differences previously mentioned render comparison among studies difficult and may explain the variations observed by different investigators. This implies that results should be interpreted with caution when analyzing studies relative to *H pylori* susceptibility testing. Nevertheless, it should be pointed out that data originating from Canadian centres show that resistance in Canada is, in general, much lower than in other areas. Antibiotics should therefore be used wisely to maintain this level as low as possible.

ACKNOWLEDGEMENTS: This study was supported by a grant from Pfizer Canada.

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