

Demonstration of Unexpected Antibiotic Resistance of Genotypically Identical *Helicobacter pylori* Isolates

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With use of multiple- and single-colony expansion procedures, the results of susceptibility testing of *Helicobacter pylori* isolates from patients with duodenal ulcer were assessed by Etest. The *H. pylori* genotype was assessed by repetitive extragenic palindrome-based polymerase chain reaction (REP-PCR). There was a high degree of genotypic heterogeneity between different patients, but a single REP-PCR pattern was found for 92% of patients. In contrast, a high degree of phenotypic heterogeneity was shown among the isolated colonies. Antibigram susceptibility patterns differed only with respect to metronidazole but not with respect to clarithromycin or amoxicillin. The 42% rate of resistance to metronidazole determined with use of the conventional multiple-strains expansion method was increased to 92% when the single-colony expansion method was used. Similarly, dual clarithromycin/metronidazole resistance was increased from 8% to 42% with single-colony expansion. Despite evidence of a single genotype in most patients, single-colony expansion shows that routine susceptibility testing may greatly underestimate the frequency of metronidazole resistance.

Helicobacter pylori is a gram-negative and microaerophilic bacterium whose normal niche is human gastric mucosa [1]. Although *H. pylori* is susceptible to many antimicrobials in vitro, clinical experience has demonstrated that *H. pylori* infection is not easy to cure in vivo. Successful treatment of *H. pylori* infection usually requires combinations of several antibiotics, often with acid suppression [2, 3]. The primary impediments to successful treatment are noncompliance with the drug regimens and antibiotic-resistant *H. pylori* [4, 5]. These findings have led to the search for successful treatment regimens. Resistance has been reported to most antimicrobials used in effective treatment protocols for *H. pylori* infection, including clarithromycin, metronidazole, tetracycline, and amoxicillin [3, 5, 6].

See editorial response by Solnick on pages 90–2.

Tremendous genetic heterogeneity exists among *H. pylori* isolates from different patients [7], and the finding of more than one genotype from a single patient has been reported [8]. A variety of methods have been used for genotyping *H. pylori*, including pulsed-field gel electrophoresis [9], random amplified polymorphic DNA (RAPD) PCR of genomic DNA [10, 11],

ribotyping [12], PCR-based restriction fragment length polymorphism analysis [13, 14], and repetitive extragenic palindrome-based PCR (REP-PCR) [7]. Molecular analysis of *H. pylori* isolates before and after therapy has proven to be very useful for distinguishing reinfection with a different strain from recrudescence [15, 16], and REP-PCR is one of the most sensitive and efficient means for distinguishing individual *H. pylori* strains [7].

The traditional protocol for antibiotic susceptibility testing is analysis by a multiple-strain (or colony) expansion method, i.e., the use of several colonies from a primary culture per biopsy per patient. *H. pylori* isolates with identical RAPD patterns have been reported to show different patterns of susceptibility to metronidazole [11, 17, 18]. The goal of this study was to investigate whether genotyping by REP-PCR gave a clue about the presence of pathogens with mixed antibiotic susceptibility in the same biopsy specimen. The REP-PCR genotypes of isolates from duodenal ulcer patients in different regions of the United States were compared with their antibiotic susceptibility profiles with regard to amoxicillin, clarithromycin, and metronidazole.

Methods

Patients and Specimens

Gastric mucosal biopsy samples were obtained from 12 patients throughout the United States who had duodenal ulcers. Biopsy specimens from the corpus of seven patients and the antrum of five patients were chosen and stored at -70°C until processed as described previously [19].

Recovery of *H. pylori* Isolates from Gastric Biopsy Specimens

Each biopsy specimen was processed and cultured as previously described [19, 20]. For multiple-colony expansion, 10–

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20 colonies were randomly picked from cultures of each biopsy specimen and mixed with sterile saline solution to a 0.5 McFarland standard, and 0.1 mL of diluted culture was subcultured onto nonselective horse blood agar (HBA) plates. For single-colony expansion, up to 10 colonies from the biopsy primary culture for each patient were picked, and each colony was subcultured as noted above on nonselective HBA plates.

After 3–4 days of incubation at 37°C in 12% CO₂ and 100% relative humidity, bacterial growth was identified as *H. pylori* on the basis of its colony morphology on plated media, gram stain reaction, and cellular morphology, as well as positive biochemical reactions to catalase, urease, and oxidase tests [21]. Growth on nonselective HBA plates was continued until adequate material was available for performing susceptibility tests. Individual cultures representing colonies from each patient were frozen at –70°C in cysteine-Albimi medium containing 20% glycerol until susceptibility testing was performed.

Preparation of Chromosomal DNA for REP-PCR

Five 100-mm HBA plates with 100% growth confluency were used to prepare DNA from each isolated colony of *H. pylori*. Bacterial cells were harvested with sterile cotton-swab applicators and washed twice by centrifugation with STE-buffer (150 mM of NaCl, 10 mM of Tris HCl, and 1 mM of EDTA; pH, 8.0) at 4°C. Chromosomal DNA from each *H. pylori* strain was prepared as described previously [22]. The DNA concentration was determined spectrophotometrically at 260 nm.

REP-PCR Amplification

The REP-PCR amplification was performed by applying repetitive sequence–based oligonucleotide primers (REP1R-Dt: 5'-IIINCNCNCATCNGGC-3'; REP2-Dt: 5'-NCGNCT-TATCNGGCCTAC-3') as previously described [7]. A negative control (without DNA template) and positive control (DNA from a clinical isolate of *H. pylori* from a patient with duodenal ulcers) were also prepared in parallel with each assay. The PCR amplicons were analyzed by 1% agarose gel electrophoresis and visualized and photographed under ultraviolet light. In order to confirm reproducibility of the REP-PCR amplification, some experiments were performed three times for each *H. pylori* isolate. Oligonucleotides were custom-synthesized (Genosys Biotechnologies Inc., The Woodlands, TX). To control for possible contamination, the same precautionary measures described previously were applied [23].

Antibiotic Susceptibility

The *H. pylori* isolates were tested for susceptibility to metronidazole (Flagyl; G.D. Searle & Co., Chicago), clarithromycin (Biaxin; Abbott Laboratories, Abbott Park, IL), and amoxicillin (Amoxil; SmithKline Beecham Pharmaceuticals, Pittsburgh) by



Figure 1. Representative REP-PCR amplification patterns of unrelated isolates of *H. pylori* analyzed by 1% agarose gel electrophoresis. Lanes 1–11, 12a, and 12b: isolates from 12 patients with duodenal ulcers; lane P, 10 ng of *Helicobacter pylori* (clinical isolate from a patient with duodenal ulcers) DNA per reaction; lane N, PCR reagents only (negative control; no DNA template); lane M contains HaeIII-digested Φ -X174 marker. Numbers at left are molecular sizes (in base pairs).

the Etest method (AB BIODISK, Solna, Sweden), according to the manufacturer's instructions and as described previously [19]. MICs were determined by the intercept of the elliptical zone of inhibition with the graded Etest strip for that antibiotic. Individual colonies growing within the clear zones of inhibition were included in the determinations of MIC values; the MIC was extrapolated by formation of an imaginary ellipse intersecting with the Etest strip, as per the instructions of the manufacturer. For the purpose of data analysis, the nonsusceptible categories of intermediate and resistant were combined into a single resistant category. Isolates were considered resistant when the MIC value was greater than the susceptible breakpoint of 8 μ g/mL for metronidazole and amoxicillin and >2 μ g/mL for clarithromycin [24].

Results

REP-PCR Banding Patterns of *H. pylori*

DNA was extracted from a total of 117 isolates (8–10 *H. pylori* isolates from each patient). As shown in figure 1, each REP-PCR produced an amplified DNA pattern of 15–20 major bands. Single *H. pylori* REP-PCR DNA patterns were found for 92% of patients (figure 1 and table 1). A pattern of two closely related *H. pylori* strains was found only for patient 12 (figure 1, lanes 12a and 12b). In contrast, great diversity was observed in *H. pylori* isolated from different patients, with the exception of isolates from unrelated patients 3 and 4, which yielded similar patterns (figure 1). The experiments were repeated at least three times without any changes in the banding

Table 1. Results of antibiotic susceptibility testing of *Helicobacter pylori* isolates obtained by multiple- and single-colony expansions.

Patient no.	No. of REP-PCR patterns	Antibiotic tested	In multiple-colony expansion [†]	Susceptibility pattern*										
				In single-colony expansion [‡] (per strain no.)										
				1	2	3	4	5	6	7	8	9	10	
1	1	M	R	R	R	R	R	R	R	R	R	R	R	R
		C	S	S	S	S	S	S	S	S	S	S	S	S
2	1	M	S	S	S	R	S	R	R	S	R	S	R	S
		C	R	R	R	R	R	R	R	R	R	R	R	R
3	1	M	S	R	R	S	S	R	R	S	R	R	R	S
		C	R	R	R	R	R	R	R	R	R	R	R	R
4	1	M	S	S	S	S	S	R	S	S	S	S	S	R
		C	R	R	R	R	R	R	R	R	R	R	R	R
5	1	M	R	R	R	R	R	R	R	S	R	R	R	R
		C	R	R	R	R	R	R	R	R	R	R	R	R
6	1	M	S	S	S	S	S	S	S	S	S	S	S	...
		C	S	S	S	S	S	S	S	S	S	S	S	...
7	1	M	S	R	S	S	R	S	R	S	S	S	S	S
		C	S	S	S	S	S	S	S	S	S	S	S	S
8	1	M	S	S	S	R	R	S	S	S	S	S	S	S
		C	S	S	S	S	S	S	S	S	S	S	S	S
9	1	M	R	R	R	S	S	R	R	S	R	R	R	S
		C	S	S	S	S	S	S	S	S	S	S	S	S
10	1	M	R	R	R	R	R	R	R	R	R	R	R	R
		C	S	S	S	S	S	S	S	S	S	S	S	S
11	1	M	S	S	R	S	R	R	R	S	R	
		C	R	R	R	R	R	R	R	R	R	
12	2	M	R	R	S	R	R	S	S	R	R	R	R	
		C	S	S	S	S	S	S	S	S	S	S	S	

NOTE. C = clarithromycin; M = metronidazole; R = resistant; REP-PCR = repetitive extragenic palindrome-based polymerase chain reaction; S = susceptible.

* Isolates were tested by Etest and were considered resistant when the MIC value was $>8 \mu\text{g/mL}$ for metronidazole and $>2 \mu\text{g/mL}$ for clarithromycin.

[†] The conventional method used to perform antibiotic susceptibility testing; 10–20 colonies picked from the primary culture were expanded.

[‡] The method used in this study; 8–10 separate colonies from the primary culture were expanded.

patterns, thus confirming the stability of the REP-PCR assay with the same isolates [7].

Antibiotic Susceptibility

The susceptibilities of the *H. pylori* isolates to metronidazole and clarithromycin is summarized in tables 1 and 2; all *H. pylori* isolates were susceptible to amoxicillin. Nine of the 11 patients (82%) with single REP-PCR patterns had more than one antibiogram (table 2). Patient 12, who had two REP-PCR patterns, also had different antibiotic susceptibility patterns.

The antibiogram patterns differed only with regard to metronidazole susceptibility. An interesting finding is that for 75% of patients the metronidazole susceptibility determined with use of the multiple-colony expansion method was the same as $>50\%$ of the susceptibilities determined with use of the single-colony expansion method (table 1). The initial designation of *H. pylori* isolates as metronidazole-resistant (for five patients, numbers 1, 5, 9, 10, and 12) or metronida-

zole-susceptible (for four patients, numbers 4, 6, 7, and 8) was always correlated with the finding of the same susceptibility in $>50\%$ of isolates. However, isolates from 3 patients were initially designated as metronidazole-susceptible, while $\geq 50\%$ of their isolates (5 of 10 for patient 2, 6 of 10 for patient 3, and 5 of 8 for patient 11) were identified by the single-colony expansion method as metronidazole-resistant.

Single-isolate expansion also revealed strains with both higher and lower MICs than were evident from multiple-colony expansion. For example, when the multiple-colony expansion method was used with the Etest, single metronidazole MICs of 4, 6, and 3 $\mu\text{g/mL}$ were observed for isolates from patients 2, 3, and 9, respectively, thus indicating susceptibility. When the single-colony expansion method was used, however, the MIC range for isolates from the same patients was 2 $\mu\text{g/mL}$ to $>32 \mu\text{g/mL}$, indicating the existence of a mixed population of metronidazole-susceptible and -resistant strains of *H. pylori* (tables 1 and 2).

Conventional testing showed dual clarithromycin and metronidazole resistance only in the isolates from patient 5. However, single-isolate expansion identified four additional patients (pa-

Table 2. MICs of metronidazole for individual strains, derived from single-colony expansion.

Patient no.	MIC ($\mu\text{g/mL}$) for indicated strain in single-colony expansion										Percentage of strains susceptible
	1	2	3	4	5	6	7	8	9	10	
2	4	4	24	4	24	32	4	32	4	12	50
3	32	32	2	4	32	32	4	32	32	3	40
4	2	4	1.5	6	32	4	2	2	2	32	80
5	32	32	32	32	32	32	3	32	32	32	10
7	32	0.2	6	12	0.2	16	8	0.1	0.1	1.5	70
8	4	4	32	16	8	4	3	4	4	4	80
9	16	32	4	2	32	32	2	32	32	3	40
11	2	32	4	32	12	32	1	32			37.5
12	32	2	32	32	2	2	32	32	32	32	30

tients 2, 3, 4, and 11) with dual clarithromycin- and metronidazole-resistant infections (table 1). The percentages of isolates from a single patient with dual resistance ranged from 20% to 90%.

Discussion

This study confirmed the reproducibility of the REP-PCR method and the high level of genetic diversity of *H. pylori* isolates from different patients [13, 25, 26]. We also confirmed that most *H. pylori* isolates from a single patient typically show only one REP-PCR pattern, and that pattern is stable during passage in the laboratory [7]. The great diversity of *H. pylori* is reflected not only by its different genotypic patterns but also by its phenotypic diversity, such as differences in drug resistance, adherence specificity, cytotoxin production, and CagA expression [5, 27–29].

Generally, *H. pylori* strains recovered from initial and follow-up biopsy specimens from the same individuals in the United States have been indistinguishable by REP-PCR or other DNA fingerprinting methods [7, 13, 18]. In contrast, mixed infection in the same patients was reported on the basis of genomic DNA patterns or antibiotic susceptibility [8, 26, 30]. Not only has more than one isolate been recovered from the stomach of a single patient [31], but more than six isolates have been recovered in sequential biopsies [17]. However, the different strains of *H. pylori* in these mixed infections were found in biopsy samples from various sites of the stomach rather than from one biopsy specimen. It is not clear how often mixed infection represents natural infection or endoscopic transmission due to poor disinfection of endoscopes and accessories [32, 33].

A standard methodology for antibiotic susceptibility testing of *H. pylori* has not been established. *H. pylori* is a microaerophile that grows slowly even under “optimal” culture conditions, and its coccoid formation (unculturable form) necessitates transfer to fresh medium when colonies are young (3–5 days old) and minute (<1 mm in diameter). Because of these

limitations, most laboratories test for antibiotic susceptibility with a multiple-colony expansion technique, i.e., 10–20 colonies are picked and transferred en masse to fresh plates. The growth is then expanded and established as a stock for that patient. Only under unusual circumstances is a single colony picked for expansion.

To address whether antibiotic resistance may be more frequent than reported with use of the routine multiple-colony expansion method of assessing antimicrobial susceptibility, we examined 8–10 colonies from primary cultures of each biopsy specimen. Only one patient had two different—yet very similar—REP-PCR DNA patterns. The high level of genomic variation among strains of *H. pylori* from different patients may be explained by the natural competence of *H. pylori* [34], allowing easy uptake of extracellular DNA, and/or may reflect the high rate of genomic recombination events that occur with this organism [35].

Although isolates from the same biopsy specimen were indistinguishable by REP-PCR fingerprint for 92% of patients, the frequency of antibiotic resistance varied remarkably. Data from the single-colony expansion showed that the frequency of antibiotic-resistant organisms was greatly underestimated by the conventional methods (table 1). For example, multiple-colony expansion predicted that only one patient was infected with combined metronidazole/clarithromycin-resistant *H. pylori*. Examination of single colonies showed that dual drug resistance was actually present in the isolates from five (45%). The finding of this pattern following treatment failure with metronidazole would be reported as development of antibiotic resistance; however, it might actually represent selection of already existing resistant strains. Hence, the metronidazole-susceptibility testing of one colony from a primary culture may be misleading, as was also shown recently by Weel et al. [11].

Therapeutic regimens including clarithromycin and metronidazole are being undermined by development of resistance [4, 36]. Our group has previously shown that the molecular mechanism of clarithromycin resistance relates to changes in the structure of the ribosome in such a way that the macrolide has markedly reduced binding to the ribosome [37]. Although

neither the mode of antimicrobial action nor the mechanism of resistance to metronidazole in *H. pylori* is understood, progress has been made in understanding the mechanism in anaerobic organisms such as *Bacteroides* and *Clostridium* species [38–42]. In these species the mode of metronidazole resistance is thought to involve reductive activation following uptake that leads to toxic effects of the reduced products in the target cell [38–42].

Perhaps similar resistance-acquiring mechanisms apply to *H. pylori*. In fact, the very wide range of MICs of metronidazole (0.125 µg/mL to >32 µg/mL) in this study suggests that resistance may result from different levels of expression of enzyme(s) responsible for the reductive activation of metronidazole in *H. pylori*. However, another potential mechanism of metronidazole resistance of *H. pylori* is perhaps due to a mutation in the genetic locus *recA* gene, as has recently been suggested by Chang et al. [43].

Although the clinical importance of metronidazole resistance is not clear, the fact that we and others [44] observed differences in only metronidazole susceptibility among single-colony expansion isolates may be a reflection of inadequate test methods [45]. Recently, it has been shown that simple anaerobic incubation eliminated metronidazole resistance in previously resistant *H. pylori* isolates [39]. Furthermore, our testing has shown that within a given test methodology, results were reproducible and intratest comparisons were valid.

Studies addressing drug-resistance mechanisms are urgently needed because antimicrobial resistance has become an increasing problem that may soon compromise the effectiveness of current therapies.

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