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Evaluation of a New Test, GenoType HelicoDR, for Molecular Detection of Antibiotic Resistance in *Helicobacter pylori*▽

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The eradication rate of *Helicobacter pylori* by standard therapy is decreasing due to antibiotic resistance, mainly to clarithromycin. Our aim was to provide a new molecular test to guide the treatment of new and relapsed cases. We first studied 126 *H. pylori* strains for phenotypic (MIC) and genotypic resistance to clarithromycin (*rrl* mutation) and levofloxacin (*gyrA* mutation) and then developed a DNA strip genotyping test on the basis of the correlation results and literature data. Clinical strains ($n = 92$) and gastric biopsy specimens containing *H. pylori* ($n = 105$) were tested blindly with the new molecular test GenoType HelicoDR. The presence of mutations or the absence of hybridization with wild-type sequences was predictive, in *rrl* for clarithromycin resistance in 91 cases (mostly the A2147G mutation) and in *gyrA* for levofloxacin resistance in 58 cases (mutations at codon 87 or 91). Genotyping revealed a mix of genotypes in 33% of the cases, reflecting a coinfection or selection for resistant mutants. The sensitivity and specificity of detecting resistance were 94% and 99% for clarithromycin and 87% and 98.5% for levofloxacin, respectively. The concordance scores were 0.96 for clarithromycin and 0.94 for levofloxacin. With global resistance rates of 46% for clarithromycin and 25% for levofloxacin, which were observed for consecutive positive biopsy specimens from 2007 and 2008, the positive and negative predictive values for detecting resistance were 99% and 94% for clarithromycin and 96% and 96% for fluoroquinolone. GenoType HelicoDR is efficient at detecting mutations predictive of antibiotic resistance in *H. pylori* when applied to strains or directly to gastric biopsy specimens.

Helicobacter pylori infection is a common chronic gastric infection worldwide with one-third prevalence (6). About 1 out of 10 humans infected with *H. pylori* suffers from various digestive diseases, such as duodenal and gastric ulcer and nonulcer dyspepsia; 1 out of 100 develops gastric adenocarcinoma; and ≤1 out of 1,000 may develop gastric mucosa-associated lymphoid tissue lymphoma. All consensus guidelines recommend eradication of *H. pylori* (6, 20) in symptomatic patients. Standard therapy combines a proton pump inhibitor, such as omeprazole, and two antibiotics, chosen from among amoxicillin, clarithromycin, and metronidazole (20). This therapy was assessed in studies in the early 1990s and demonstrated an eradication rate of *H. pylori* of over 80%. However, the eradication rate is decreasing, with as low as 60% success in some countries, and this is related to the increase in clarithromycin and metronidazole resistance reported worldwide (9, 10, 17). Fluoroquinolones, such as levofloxacin and moxifloxacin, are often used for rescue therapy in a third- or fourth-line treatment (20, 31).

Antibiotics used for the treatment of *H. pylori* infection are usually not chosen on the basis of routine susceptibility testing, because *H. pylori* is a fastidious microorganism requiring 3 to 10 days in a microaerobic atmosphere, and susceptibility results are not reliable for all antibiotics (17, 22). Indeed, susceptibility breakpoints have been difficult to set due to the lack of standard methods for susceptibility testing and difficulties in assessing the correlation between susceptibility results and clinical outcomes. Phenotypic resistance is correlated with clinical and microbiological failure for clarithromycin, but not for metronidazole (21). The eradication rate drops from 88% in the case of a clarithromycin-susceptible strain to less than 20% in the case of clarithromycin resistance (7, 21). Fluoroquinolone resistance was also shown to be correlated with treatment failure (24). Because resistance rates vary according to the country and patient characteristics, the choice of antibiotics on the basis of susceptibility results might be an effective strategy to improve *H. pylori* eradication. Since susceptibility testing is cumbersome, molecular methods for detection of resistance may be cost-effective.

The mutations leading to resistance are now well known for macrolides and fluoroquinolones, although they are still unclear for metronidazole and amoxicillin. Clarithromycin resistance in *H. pylori* is due to point mutations in the *rrl* gene encoding the 23S rRNA, with three major mutations described: A2146C, A2146G, and A2147G (the numeration is from genome sequencing of NC000921 and NC000915, positions 2146 and 2147, formerly described as 2142 and 2143 [reviewed in references 21 and 22]). The resistance of *H. pylori* to quinolones is due to point mutations in the so-called quinolone resistance-determining region of the *gyrA* gene coding

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for the A subunit of the DNA gyrase, mainly at codons 87 and 91 (corresponding to 83 and 87 in *Escherichia coli* numbering) (1, 4, 23, 30).

Our objective was to develop and implement a molecular method to easily detect mutations predictive of clarithromycin and fluoroquinolone resistance in *H. pylori*. We based our test on the DNA strip methodology used with success for other pathogens, such as *Mycobacterium tuberculosis* (13). We first designed a prototype test using a panel test of 126 *H. pylori* strains for which the MICs of clarithromycin and fluoroquinolones and the *rrl* and *gyrA* genotypes had been determined. Then, the new test was applied blindly to clinical strains and gastric biopsy specimens containing *H. pylori*, and the results were compared to those of susceptibility testing done routinely. The specificity of the new test for *H. pylori* was evaluated by testing strains of *Helicobacter* species other than *H. pylori*, as well as negative biopsy specimens. The new test was concordant with reference tests for 94 to 98% of the samples, either performed on isolated strains or directly on gastric biopsy specimens containing *H. pylori*, and was easy to perform.

MATERIALS AND METHODS

***H. pylori* strains and gastric biopsy specimens containing *H. pylori*.** The first phase of the study compared phenotypic and genotypic profiles of resistance to clarithromycin and to fluoroquinolones (levofloxacin and ciprofloxacin) for 126 strains isolated from March 2004 to September 2005 at Henri Mondor Hospital, Créteil, France. The second phase studied 92 *H. pylori* strains and 105 gastric biopsy specimens positive for *H. pylori*. They were consecutive positive samples obtained either at Henri Mondor Hospital (67 strains and 74 biopsy specimens) or at the National Reference Center at Bordeaux Hospital (25 strains and 31 biopsy specimens) in 2006, 2007, and 2008. In the two hospitals, gastric biopsy specimens were routinely cultured and subjected to real-time PCR for *H. pylori* detection, and *H. pylori*-positive biopsy specimens were subjected to *rrl* mutation detection as described previously (18, 27).

Phenotypic susceptibility testing. Susceptibility to clarithromycin and to fluoroquinolones (levofloxacin or ciprofloxacin, since we had previously shown that the results were concordant [4]) was assessed using the Etest method (AB Biodisk, Solna, Sweden) performed as previously described (4, 9) and using susceptibility breakpoints of $\leq$0.5 μg/ml for clarithromycin and $<$1 μg/ml for ciprofloxacin or levofloxacin (http://www.clsi.org and http://www.eucast.org). *H. pylori* NCTC11637 was used as a control. Briefly, a bacterial inoculum was prepared in brain heart infusion broth from subcultures grown on Pylori agar (bioMérieux, Marcy l'Etoile, France) so that the turbidity was equal to that of a 3 McFarland standard. Mueller-Hinton agar supplemented with 10% sheep blood and prepared extemporaneously was inoculated and incubated at 37°C under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) for 72 h.

DNA isolation and PCR sequencing of *H. pylori* strains and gastric biopsy specimens. Genomic DNA of strains was extracted using the QIAmp DNA Mini Kit (Qiagen, Courtaboeuf, France). Total DNA from biopsy specimens was extracted using the High Pure PCR template kit after following the protocol for tissue lysis (Roche Diagnostics, Switzerland). Before DNA extraction, gastric biopsy specimens (from the antrum and corpus) were crushed or cut into pieces and mixed with 0.5 ml of brain heart infusion broth.

DNA regions involved in clarithromycin (*rrl* gene) and fluoroquinolone (*gyrA* gene) resistance were amplified by PCR as previously described (4, 22). Typical reaction mixtures (50 μl) contained 1× reaction buffer, 1.5 mM of $MgCl_2$, 200 μM of deoxynucleotide triphosphates, 1 μM of each primer (Proligo, France), 1.25 U of *Taq* polymerase (Q-Biogene, Illkirch, France), and 150 ng of DNA template. PCR-amplified fragments were purified by using Montage PCR centrifugal filter devices (Millipore, Molsheim, France) and sequenced by the dideoxy chain termination method with the ABI Prism BigDye Terminator v3.1 cycle-sequencing kit (Applied Biosystems, Courtaboeuf, France). The oligonucleotide primers used for DNA sequencing were those used for PCR. The nucleotide and deduced amino acid sequences were analyzed with Seqscape v2.0 software (Applied Biosystems).

GenoType HelicoDR testing. Genomic DNA of the *H. pylori* strains of the phase 2 panel and total DNA extracted from the gastric biopsy specimens were subjected blindly to DNA strip testing. The strips were coated at the Hain Lifescience factory (Nehren, Germany) with different specific oligonucleotides (DNA probes) using the DNA strip technology. The probes were designed to hybridize with the sequences of the wild-type alleles (WT probes) or the mutated alleles (MUT probes).

Amplification, hybridization, and interpretation were performed in a procedure similar to those for other GenoType tests (12). Briefly, 35 μl of 5′-biotinylated primers and nucleotide mixture, 5 μl of polymerase buffer, 2 μl of 25 mM $MgCl_2$ stock solution, 3 μl of water, and 5 μl of total DNA (20 to 100 ng) were mixed with 1 U of Hot Star *Taq* polymerase (Qiagen) per reaction mixture. The PCR run comprised 30 cycles for strains and 35 cycles for biopsy specimens. In both protocols, the denaturation cycle was 1 cycle at 95°C for 15 min, followed by 10 cycles at 95°C for 30 s and at 58°C for 2 min. Then, 20 cycles (strain) or 25 cycles (biopsy specimen) were composed of a first step at 95°C for 25 s, a second step at 53°C for 40 s, and a third step at 70°C for 40 s. The PCR ended with 8 min at 70°C. Hybridization was performed using the TwinCubator at a temperature of 45°C. The denaturation solution was mixed with 20 μl of the amplified sample and submitted to the usual protocol for hybridization (12, 13).

In order to assess positive and negative bands, the DNA strips were stuck on an evaluation sheet after the hybridization, and a template was aligned side by side with the conjugate control band of the respective strip. Control bands that should appear positive to validate the test were bands corresponding to the conjugate control and the amplification control, an identification band for *H. pylori* (the PCR fragment was from the *rrl* gene, which codes for the 23S rRNA, as stated above), and amplification controls of the *rrl* and *gyrA* genes.

The analytical sensitivity of the GenoType HelicoDR test for the detection of *H. pylori* was tested by comparing the positivity of the *H. pylori* detection probe and dilutions of bacterial suspensions. The analytical specificity of the GenoType HelicoDR test was determined by subjecting to the test DNA extracted from reference strains of 20 *Helicobacter* species other than *H. pylori*. In addition, 23 biopsy specimens known to be negative for *H. pylori* were also tested for specificity.

RESULTS

Concordance between phenotypes and genotypes. The clarithromycin, levofloxacin, and ciprofloxacin MICs for all 126 strains included in the phase 1 study were determined, and *rrl* and *gyrA* resistance-determining regions were sequenced. The results are detailed in Table 1. For the strains and biopsy specimens included in the phase 2 study, clarithromycin susceptibility was compared to the *rrl* mutation detection by real-time PCR, since the molecular detection is done routinely. Determination of *gyrA* mutations was not done routinely. PCR sequencing of *rrl* or *gyrA* was done in cases of discord between the susceptibility data and the results of the GenoType HelicoDR test.

GenoType HelicoDR probe design according to the sequencing of strains from phase 1. The MUT and WT probes were designed from the mutations observed in the resistant strains from the phase 1 study; mutations in the *rrl* gene encoding the 23S rRNA for the clarithromycin-resistant strains and mutations in the *gyrA* gene for the fluoroquinolone-resistant strains. These results were gathered with those described in the literature (reviewed in reference 22). The probes are listed in Table 2. In *rrl*, the most prevalent mutations were A2147G (92% in our study and 54% in the literature), A2146G (8% in our study and 28% in the literature), and A2146C (no strains in our study but 4% in the literature). *gyrA* mutations have been previously described and compared to those in the literature (4). Overall, in *gyrA*, the mutations were distributed at codons 87 and 91 with the following prevalences: N87K, 27% in our study and 41% in the literature; T87Y, 9% and 10%; D91N, 36% and 30%; D91Y, 14% and 15%; D91G, 0% and 11%; and D86N, 4% and 2%. By sequencing the *gyrA* quinolone resistance-determining region in fluoroquinolone-susceptible strains, we

TABLE 1. Concordance between phenotype (MIC determined by Etest) and genotype (sequencing or real-time PCR) for *H. pylori* strains included in the study

H. pylori[a]	*n*	*rrl* genotype			*gyrA* genotype		
		Wild type	Missense mutation	No data	Wild type	Missense mutation	No data
Phase 1							
Development panel *H. pylori* strains	126						
CLA-R	40	3[b]	37				
CLA-S	86	86	0				
FQ-R	21				0	21	
FQ-S	105				105[c]	0	
Phase 2							
Clinical *H. pylori* strains	92						
CLA-R	48	2	34	12			
CLA-S	44	21	1	22			
FQ-R	41						41
FQ-S	51						51
Gastric biopsy specimens	105						
CLA-R	49	0	49				
CLA-S	56	56	10				
FQ-R	26						26
FQ-S	79						79

[a] CLA, clarithromycin; FQ, fluoroquinolone; S, susceptible; R, resistant.
[b] Clarithromycin MIC, 2 μg/ml.
[c] Sixteen strains harbored a Thr87, which confers fluoroquinolone susceptibility.

TABLE 2. Probes hybridized on the DNA strip of the GenoType HelicoDR test for detection of mutations in the *gyrA* and the *rrl* genes[a]

Probe	Codon	Nucleotides	Associated phenotype[b]
gyr87-WT1	N87	AAC	FQ-S
gyr87-WT2	N87	AAT	FQ-S
gyr87-WT3	T87	ACC	FQ-S
gyr87-WT4	T87	ACT	FQ-S
gyr87-MUT	N87K	AAA	FQ-R
gyr91-WT	D91	GAT	FQ-S
gyr91-MUT1	D91N	AAT	FQ-R
gyr91-MUT2	D91G	GGT	FQ-R
gyr91-MUT3	D91Y	TAT	FQ-R
23S-WT	2146 and 2147	AA	CLA-S
23S-MUT1	2146	A2146G	CLA-R
23S-MUT2	2146	A2146C	CLA-R
23S-MUT3	2147	A2147G	CLA-R

[a] Numbering system used in *H. pylori* J99 and 26695 (GenBank accession no. NC000921 and NC000915).
[b] CLA, clarithromycin; FQ, fluoroquinolone; S, susceptible; R, resistant.

observed an Asn (N)/Thr (T) polymorphism at codon 87, with 15% of the strains with T87. It was then necessary to include four wild-type probes corresponding to codon 87 (two for Asn87 and two for Thr87) in *gyrA* in the DNA strip, as shown in Table 2 and Fig. 1.

GenoType HelicoDR results for detection of clarithromycin resistance for the nondevelopmental strains and specimens (phase 2 study). There were 197 results of testing by the GenoType HelicoDR test for the 92 clinical strains and the 105 gastric biopsy specimens containing *H. pylori* in phase 2. Representative strips are shown in Fig. 2. Several times (see Heterogeneity of the *H. pylori* population below), more than one mutation was detected per strain or biopsy specimen. One *rrl* mutation was observed in 91 strains or biopsy specimens, but 100 "cases" of *rrl* mutation were reported overall (for the observed genotypes, see Table 4). Overall, the most frequent mutation was A2147G (MUT3 profile), observed in 35 strains and 41 biopsy specimens (76% of the mutated alleles), followed by A2146G (MUT1 profile) in 12 strains and 8 biopsy specimens (20%) and A2146C (MUT2 profile) in 1 strain and 2 biopsy specimens (3%).

The concordance between the results of the GenoType HelicoDR test and the results of genotyping by real-time PCR was 99.5%. The discordant case showed a wild-type band at the *rrl* locus and showed a mixture of wild-type and mutated alleles by real-time PCR, which was in accordance with the observation of resistance (MIC, 256 μg/ml). The concordance between the results of the GenoType HelicoDR test and clarithromycin susceptibility (phenotype) is presented in Table 3. Of the seven discordant cases, five were due to the mixing of susceptible bacteria with a wild-type *rrl* genotype and resistant bacteria with a mutated *rrl* genotype (see Heterogeneity of the *H. pylori* population below).

The sensitivity and specificity for the detection of a clarithromycin-resistant strain were 94% and 99%, respectively, and the concordance score was 0.96 for clarithromycin. According to the global prevalence of *H. pylori* resistance to clarithromycin observed in the phase 2 study, which included consecutive strains and positive biopsy specimens (46% for biopsy specimens and strains together), the positive and negative predictive values of the GenoType HelicoDR test for detecting clarithromycin resistance were 99% and 94%, respectively.

GenoType HelicoDR results for levofloxacin resistance detection. There were 197 results given by GenoType HelicoDR for *gyrA* genotyping, with examples of strips shown in Fig. 2. For each *H. pylori* strain, the results for the two codons, 91 and 87, have to be taken into account in order to classify it as a wild-type genotype or a mutated genotype. As for *rrl* mutation detection, several times (see Heterogeneity of the *H. pylori* population below) more than one genotype was detected per strain or biopsy specimen.

A mutated *gyrA* genotype was observed in 60 *H. pylori* strains (30%): 30 with one mutation at codon 87; 25 with one mutation at codon 91; and 4 with a *gyrA* mutation at both codons 87 and 91, 1 of which had two mutations at codon 91; and the remaining 1 with two mutations at codon 91. Detailed results are presented in Table 4. The most frequent mutation was D91N (MUT1), followed by the T87I mutation. This mutation was detected as "no wild-type band and no MUT band" at codon 87, and the mutation was assessed by PCR sequencing.

In the remaining two cases of no band detection at codon 87, N87Y was observed in one case. For the remaining strain, the modified codon was not codon 87 but codon 88, where the wild-type triplet was GCA instead of GCG. This probably led to weaker hybridization with the oligonucleotides targeting codon 87.

Probing with the four wild-type alleles at codon 87 showed that the preponderant codon was that of the wt1 profile (AAC), followed by wt2 (AAT), wt3 (ACC), and wt4 (ACT)

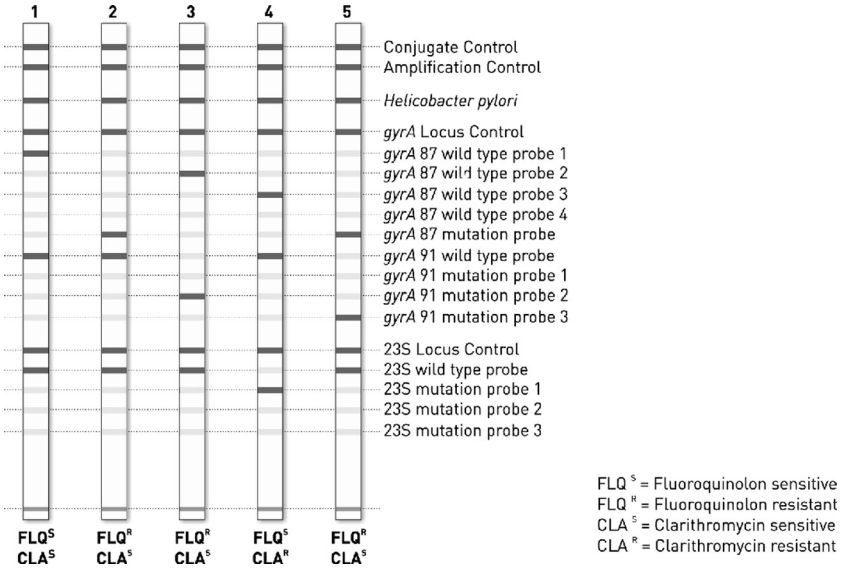

FIG. 1. Prototype of the strip of the GenoType HelicoDR test. Lane 1, *gyrA* pattern with a wt1 codon at position 87, a wt codon at codon 91, and a wild-type *rrl* pattern; lane 2, *gyrA* pattern with a MUT codon at position 87, a wt codon at codon 91, and a wild-type *rrl* pattern; lane 3, *gyrA* pattern with a wt2 codon at position 87, a MUT2 codon at position 91, and a wild-type *rrl* pattern; lane 4, *gyrA* pattern with a wt3 codon at position 87, a wt codon at position 91, and a MUT1 *rrl* mutation; lane 5, double mutation in *gyrA* with mutation at both 87 (MUT) and 91 (MUT3) and a wild-type *rrl* pattern.

(Table 4). Overall, threonine codon 87 (wt3 or wt4 triplet) was present for 35 *H. pylori* strains detected, which represented 25% of the *H. pylori* strains with a wild-type *gyrA* allele.

The concordance between the GenoType HelicoDR test and fluoroquinolone susceptibility, defined by either ciprofloxacin or levofloxacin susceptibility, is presented in Table 5. The discordant results were mainly a wild-type allele with a high MIC (nine discordant results). For the two biopsy specimens for which a mutated allele was detected in a fluoroquinolone-susceptible *H. pylori* strain, there was no band hybridizing at codon 87 in one case (sequencing revealed a silent mutation at codon 88, as described above) and no band at codon 91 in the second case. By repeating the test, we observed weak hybridization to the WT91 probe, and sequencing confirmed a wild-type *gyrA* allele.

The sensitivity and specificity for detecting resistance were 87% and 98.5%, respectively, and the concordance score was 0.94.

According to the 25% prevalence of levofloxacin resistance in our study, positive and negative predictive values were 96% and 96%, respectively.

Heterogeneity of the *H. pylori* population. In 66 (33%) of the cases (30 strains and 36 biopsy specimens), more than one hybridization band was observed at one codon. This indicated that there were at least two alleles amplified during the PCR. The mixture was shown with the *rrl* probes or the *gyrA* probes.

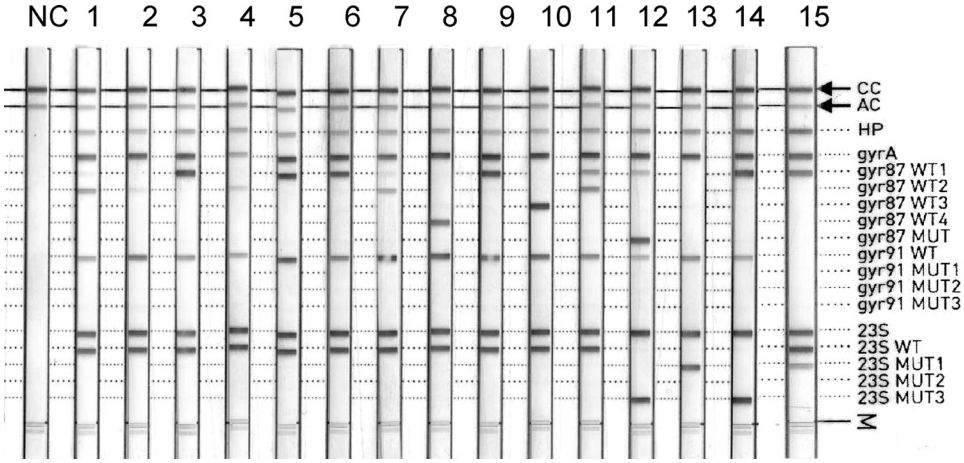

FIG. 2. Representative GenoType HelicoDR DNA strip results obtained with *H. pylori* strains and biopsy specimens showing (i) *rrl* alleles that are wild type (lanes 1 to 11) or mutated (12 to 15), (ii) *gyrA* alleles that are wild type (lanes 1, 3 to 11, and 15) or mutated (a mutation at codon 87 in lane 12 and no hybridization with probes of codon 87 in lane 2), and (iii) heterogeneity of the *H. pylori* population (lanes 7, 11, 12, and 15). NC, negative control; CC, conjugate control; AC, amplification control.

TABLE 3. Concordance for clarithromycin results between GenoType HelicoDR and susceptibility testing for the *H. pylori* included in the phase 2 study

Reference results[a]	GenoType HelicoDR result		
	n	Mutation allele[b]	Wild-type allele[c]
Strains (n = 92)			
Cla resistant	48	43	5[d]
Cla susceptible	44	1[e]	43
Biopsy specimens (n = 105)			
Cla resistant	49	48	1[f]
Cla susceptible	56	0	56
Total (n = 197)			
Cla resistant	97	91	6
Cla susceptible	100	1	99

[a] Reference phenotype (Etest MIC using a breakpoint of 0.5 μg/ml).
[b] At least one of the following mutation bands: 23S-MUT1, 23S-MUT2, or 23S-MUT3.
[c] Presence of the 23S-WT band and no 23S mutation band.
[d] For three strains and one biopsy specimen, a mixture of wild-type and resistant populations was assessed; for the two remaining strains, the clarithromycin MICs were 6 and 24 μg/ml, respectively.
[e] A MIC of 0.016 μg/ml and a double-genotype population were assessed by real-time PCR.
[f] Clarithromycin MIC of 1 μg/ml.

TABLE 4. Details of genotypes detected by the GenoType HelicoDR test for the *rrl* or *gyrA* gene in *H. pylori* strains and gastric biopsy specimens in the phase 2 study

Genotype	No. in clinical strains (n = 92)	No. in biopsy specimens (n = 105)
23S rRNA gene (*rrl*)		
WT	48	57
MUT1	6	5
MUT3	25	34
WT + MUT1	2	1
WT + MUT3	5	5
WT + MUT1 + MUT3	1	0
WT + MUT1 + MUT2	0	1
WT + MUT2 + MUT3	1	1
MUT1 + MUT3	3	1
gyrA gene		
Codon 87		
WT1	34	41
WT2	8	11
WT3	15	8
WT4	2	6
WT1+WT2	15	20
WT2+WT4	1	0
WT3+WT4	0	2
WT1+WT2 + WT3	0	1
WT 1 + MUT	2	3
MUT	7	2
WT1+WT2 + MUT	1	0
No WT or MUT band	7	11
Codon 91		
WT	73	93
MUT1	6	5
MUT2	4	3
MUT3	1	1
WT + MUT1	4	0
WT + MUT3	1	0
WT + MUT2	2	1
WT + MUT1 + MUT2	1	0
WT + MUT1 + MUT3	0	1
No WT or MUT band	0	1

WT, wild-type allele; MUT, mutated allele (see Table 2 for the codon sequences).

It concerned a resistant and a wild-type sequence, as well as a mixture of two wild-type sequences, but with different triplets for the same amino acid. The results are presented in Table 6, and examples are shown in Fig. 2.

Analytical sensitivity and specificity with the GenoType HelicoDR test for the detection of *H. pylori* DNA. All biopsy specimens detected as positive for *H. pylori* by the real-time PCR detection method and by culture were positive with GenoType HelicoDR. Bacterial suspensions of *H. pylori* strains with a McFarland density of 1.0 to 8.0 (DNA concentration, 3.6 to 21.4 μg/ml, respectively) gave a positive signal with the GenoType HelicoDR test.

The analytical specificity of the GenoType HelicoDR test was measured by testing DNA extracted from 20 *Helicobacter* species other than *H. pylori*. The point results of hybridization are shown in Table 7. Although some amplicons hybridized to some probes (*rrl* probes more often than *gyrA*), none of the *Helicobacter* species hybridized to the *H. pylori* control probe or to all of the WT or MUT probes, except *Helicobacter acinonychis*, which is known to be the closest to *H. pylori*.

For the 23 gastric biopsy specimens that were negative for *H. pylori* by culture, molecular detection (real-time PCR), and histology, GenoType HelicoDR results were negative for the *H. pylori* control band, except for one biopsy specimen. Other bands were also observed for the *gyrA* and the *rrl* control probes and the *rrl* alleles (WT and MUT1 plus MUT3). The gastric histology results for the patient with the positive biopsy showed atrophy and inflammation in the antrum but no *H. pylori*. This suggests that the biopsy specimen may have contained *H. pylori* DNA, even though other reference methods of detection (culture and real-time PCR) were negative. For seven biopsy specimens, although some bands were positive (mostly the *rrl* control probe), the absence of the *H. pylori* control band and of most of the control bands led to a negative result.

DISCUSSION

Because the success of *H. pylori* eradication treatment is closely related to prior recognition of antibiotic resistance and because susceptibility testing is rarely done due to fastidious growth of the bacterium, we developed and evaluated a new molecular test that rapidly detects antibiotic resistance in *H. pylori*. The test, based on DNA strip technology, was developed to be applicable to strains, as well as to gastric biopsy specimens, and to be affordable and easy to perform in clinical microbiology laboratories.

The detection of clarithromycin and fluoroquinolone resistance was targeted, since (i) clarithromycin and levofloxacin are both effective drugs to treat *H. pylori* infection (6) and (ii) resistance to these drugs has been clearly shown to be correlated with eradication failure (21). Since *H. pylori* resistance

TABLE 5. Concordance of fluoroquinolone results between GenoType HelicoDR and susceptibility testing for the *H. pylori* strains included in the phase 2 study

Phenotype[a]	GenoType HelicoDR result		
	n	Mutation allele[b]	Wild type[c]
Strains (n = 92)			
FQ resistant	41	34	7[d]
FQ susceptible	51	0	51
Biopsy specimens (n = 105)			
FQ resistant	26	24	2[e]
FQ susceptible	79	2[f]	77
Total (n = 197)			
FQ resistant	67	58	9
FQ susceptible	130	2	128

[a] Ciprofloxacin or levofloxacin susceptibility testing using breakpoint of 1 μg/ml. FQ, fluoroquinolone.
[b] One MUT band or no WT band at codon 87 or at codon 91.
[c] Presence of at least one WT band at codons 87 and 91 and no MUT band.
[d] MIC, 32 μg/ml for six strains and 8 μg/ml for one strain.
[e] MIC, 32 μg/ml for the two strains.
[f] MIC < 0.5 μg/ml; silent mutation at codon 88 for one biopsy specimen and absence of wild-type codon 91 for the second.

TABLE 6. Heterogeneity of the *H. pylori* population per patient as assessed by Genotype HelicoDR

Heterogeneity	No. (%) in strains (n = 92)	No. (%) in gastric biopsy specimens (n = 105)	Total (%) (n = 197)
No heterogeneity	62 (67)	69 (66)	131 (66.5)
Heterogeneous genotypes[a]	30 (33)	36 (32)	66 (32)
Wild-type and resistant *rrl* alleles	9 (10)	8 (8)	17 (9)
Several resistant *rrl* alleles	5 (5)	2 (2)	7 (3.6)
Wild-type and resistant *gyrA* alleles	11 (12)	5 (5)	16 (8)
Several wild-type *gyrA* alleles	17 (18.5)	23 (22)	40 (20)

[a] At least two genotypes (details of genotypes are listed in Table 4).

mechanisms for these drugs are due to point mutations in a small region of one gene, it was possible to design a DNA strip test based on the few oligonucleotides detecting mutations. Detection of metronidazole resistance, although the most frequent, could not be included in the test, since it involves several genes (*rdxA*, *frxA*, and *fdxB*) without clear correlation with the resistance phenotype so far (14, 21).

Clarithromycin resistance is correlated with *rrl* mutations (A2146G, A2146C, or A2147G) for more than 90% of the clarithromycin-resistant clinical strains (21). Few mutations have been described outside the two positions 2146 and 2147, and their association with clarithromycin resistance is not yet consensual (3, 16, 28, 32). By using the GenoType HelicoDR test, we showed that 94% of the clarithromycin-resistant *H. pylori* strains harbored one of the three mutations listed above (37 out of the 40 strains in the development study and 91 out of 97 *H. pylori* strains in the evaluation study).

Since 1995, fluoroquinolone resistance in *H. pylori* has been related to *gyrA* gene mutations at positions 87 and 91 (*H. pylori* numbering system) (17, 23, 30). Fluoroquinolone (levofloxacin, gatifloxacin, and ciprofloxacin) resistance was correlated with *gyrA* mutations in 80 to 100% of the fluoroquinolone-resistant strains (1, 4, 15, 25). The mutations are generally found in codons 87 and 91 and rarely in codon 86 (4). This is concordant with the present results of GenoType HelicoDR testing in 67 fluoroquinolone-resistant *H. pylori* strains, 87% of which showed a mutation either at codon 87 (57% of the mutations) or at codon 91 (48%). In some cases, the GenoType HelicoDR results showed a pattern concordant with a resistant allele but with no band corresponding to a precise mutation, and also without any wild-type-positive band. In most of the cases, sequencing showed a missense mutation at codon 86 or 87 that was not specifically present on the DNA strip. Similar observations were made for other GenoType strips, such as the GenoType MTBDR*plus* strip (13). Thus, the test can detect a broad spectrum of mutations mediating resistance, and not only those present as mutated alleles on the strip. However, control sequencing is recommended when no band is seen, since in one case in our study, the *gyrA* sequence showed a silent mutation.

The results of the GenoType HelicoDR test were in concordance with those of clarithromycin and fluoroquinolone susceptibility testing. The discordances observed were mainly due to discrepancies between the genotyping and phenotyping methods, especially for fluoroquinolone. There was agreement of the GenoType HelicoDR test with genotyping performed by another method (PCR sequencing or real-time PCR). Susceptibility testing methods, although standardized, were not demonstrated to be fully reliable for bacteria such as *H. pylori* because of its fastidious growth and difficulties in assessing an adequate inoculum (9).

In our study, we showed that more than 30% of the *H. pylori* strains present in gastric biopsy specimens or isolated as strains were in fact mixtures of several genotypes. The presence of more than one genotype in a single biopsy specimen has been shown to be the consequence of either different strains coexisting in the gastric mucosa or the presence of mutated and wild-type alleles in the same strain (26, 32). Using culture, a similar proportion of 20 to 25% of the *H. pylori* cases was found. When a mixture of susceptible and resistant genotypes has been found, it has been related to clinical failure so far (26). In some cases, the results of genotyping by our test showed a mixture of wild-type alleles. Recently, Sheu et al. showed that infections with more than one strain are associated with more severe disease (29).

Other genotyping methods have been developed for the detection of antibiotic resistance in *H. pylori* based on the LiPA technology (32, 33), real-time PCR (2, 18, 19, 27), fluorescence in situ hybridization (11), microarray (5, 35), or others (34). Most of them are in-house tests that are not commercially available, and they detect only clarithromycin resistance. This may explain why detection of antibiotic resistance is rarely implemented in microbiology laboratories. However, there is a need to diagnose resistance in developed countries, where ulcers and gastric cancers are still frequent, as well as in developing countries, where most of the population is infected and 1/10 suffer from gastroduodenal ulcers.

TABLE 7. Results of the Genotype HelicoDR test for strains of *Helicobacter* species other than *H. pylori*

Non-*pylori* *Helicobacter* species	Hybridization results for the probes present on the DNA strip[a]															
	HP	gyrA	gyr87 WT1	gyr87 WT2	gyr87 WT3	gyr87 WT4	gyr87 MUT	gyr91 WT	gyr91 MUT1	gyr91 MUT2	gyr91 MUT3	23S	23S WT	23S MUT1	23S MUT2	23S MUT3
H. winghamensis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H. cholecystus	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+
H. mustelae	−	+	−	−	−	−	−	+	±	−	−	+	+	−	−	−
H. cinaedi	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
H. canadensis	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−
H. fennelliae	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−
H. acinonychis	+	+	+	+	−	−	−	+	−	−	−	+	+	−	−	−
H. hepaticus	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H. canis	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
H. salomonis	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
H. pametensis	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
H. muridarum	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
H. rappini	−	−	−	−	−	−	−	−	−	−	−	±	+	−	−	−
H. bizzozeronii	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
H. ganmani	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H. pullorum	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H. felis	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
H. bilis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H. typhlonius	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
H. cetorum	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−

[a] +, positive signal; −, negative signal; ±, weakly positive signal.

We developed a test that is easy to perform and that is based on the worldwide experience of *M. tuberculosis* testing (GenoType MTBDR*plus* kit) (12). It requires a thermocycler, which nowadays is present in most laboratories, and a specific incubator for hybridization. Although we did not evaluate the quantity of bacteria necessary to obtain a positive GenoType HelicoDR test result, the analytical sensitivity with biopsy specimens was not lower than that with real-time PCR or culture, since we tested biopsy specimens known to be *H. pylori* positive by these methods, and in all cases the GenoType HelicoDR test led to a positive result. Since the GenoType HelicoDR test has not been tested on feces, we do not recommend using it, especially because the presence of large amounts of other bacteria may hamper the specificity of the multiplex PCRs and of the hybridization.

Despite the recommendation for antibiotic treatment of *H. pylori* infection since 1995 (6, 20) and the increase in antibiotic resistance, susceptibility testing of *H. pylori* is rarely done and is limited to a few laboratories with expertise. Moreover, it is recommended only for patients suffering from their second relapse. Although phenotypic susceptibility testing was performed in the 1990s, it was for the most part abandoned because of the fastidious growth of the organism and also because of a small impact at that time on the treatment and outcome. Now, since primary antibiotic resistance has reached over 20% for clarithromycin, it is difficult to avoid performing the detection of antibiotic resistance even in new cases of *H. pylori* infection. Clarithromycin is still the first-line therapy recommended worldwide. Since the GenoType HelicoDR test accurately predicts clarithromycin resistance, it can be used to decide the first-line treatment without facing the 80% failure observed in clarithromycin-resistant cases (7). This will allow the development of new strategies of antibiotic therapy based on susceptibility tests, and it will improve *H. pylori* eradication rates, which have decreased to 65 to 70% in the last few years. In relapse cases, where the rate of resistance to fluoroquinolones is increasing (4, 8, 25), the detection of fluoroquinolone resistance will provide new information for the choice of antibiotics to be used in third-line therapies, often a combination of amoxicillin with levofloxacin or gatifloxacin, tetracycline, or rifabutin; resistance to the last two antibiotics is still very rare.

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