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Accurate Prediction of Macrolide Resistance in *Helicobacter pylori* by a PCR Line Probe Assay for Detection of Mutations in the 23S rRNA Gene: Multicenter Validation Study

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***Helicobacter pylori* strains from 299 patients were tested in six laboratories in different countries. Macrolide susceptibility of the strains was determined by agar dilution (17.4%) or the epsilometer test (82.6%). Mutations in the 23S ribosomal DNA (rDNA) that are associated with macrolide resistance were analyzed by PCR and reverse hybridization (PCR-line probe assay [LiPA]). This method identifies A2115G, G2141A, A2142G, A2142C, A2142T, A2143G, and A2143C mutations in the 23S rDNA. *vacA* s-region (s1a, s1b, s1c, and s2) and m-region (m1, m2a, and m2b) genotypes and *cagA* status were also determined using another PCR-LiPA system. Of the 299 strains investigated by MIC testing, 130 (43.5%) were resistant and 169 (56.5%) were susceptible to clarithromycin. Of the 130 resistant strains, 127 (97.7%) contained 23S rDNA mutations, whereas 167 (98.8%) of the 169 susceptible strains contained wild-type sequences. The predominant mutations were A2143G (45.2%) and A2142G (33.3%). Twenty-eight (19.8%) strains contained multiple 23S rDNA mutations. Only five resistant strains contained the A2142C mutation (three of these in combination with the A2142G mutation), and the A2115G, G2141A, A2142T, and A2143C mutations were not found. MICs of clarithromycin for the A2142G mutant strains were significantly higher than MICs for the A2143G strains. Although there was no significant association between 23S rDNA mutations and the *vacA* and *cagA* status, clarithromycin-susceptible strains more often contained mixed *vacA* genotypes, indicating the presence of multiple *H. pylori* strains. In conclusion, our data confirmed the very strong association between 23S rDNA mutations and macrolide resistance and showed that the PCR-LiPA permits accurate and reliable diagnosis of macrolide resistance in *H. pylori*.**

Helicobacter pylori is a gram-negative bacterium that colonizes the human stomach. Persistent infection with *H. pylori* is associated with chronic gastritis and peptic ulcer disease and may eventually result in the development of atrophic gastritis, mucosa-associated lymphoid tissue lymphoma, and gastric cancer (1, 7).

Infection with *H. pylori* can be effectively treated by a combination of a proton pump inhibitor and/or an H_2-receptor antagonist with multiple antibiotics. Metronidazole, amoxicillin, clarithromycin, and tetracycline are frequently included in triple or quadruple regiments (4, 21). Resistance to antimicrobial agents has a significant impact on the efficacy of anti-*Helicobacter* treatment. Resistance to metronidazole is observed in 10 to 50% of the cases in developed countries but can be as high as 90% in developing countries (15). The prevalence of macrolide-resistant strains varies among countries and ranges from less than 2% of the strains in The Netherlands and Norway (16, 27) to more than 10% in France and some other countries (2, 5, 15, 18). Moreover, the prevalence of resistant strains appears to be increasing (5, 8, 12, 20). Resistance to clarithromycin decreases the effectiveness of antibiotic therapy by an average of 55% (6, 10).

The major cause of macrolide resistance in *H. pylori* is the lack of binding of the macrolides to the 23S rRNA components of the bacterial ribosome due to modification of the target site by methylation or point mutations in the peptidyltransferase region of domain V of the 23S rRNA (30). *H. pylori* contains two copies of the 23S ribosomal DNA (rDNA) gene, and five distinct point mutations have been reported that are associated with macrolide resistance in natural *H. pylori* strains, i.e., G2115→A, G2141→A, A2142→G, A2142→C, and A2143→G (11, 19, 29).

Since macrolide resistance is clinically important, and because the prevalence of resistant strains is increasing, there is a clear need for rapid and accurate diagnostic methods to determine macrolide resistance. The conventional method to determine the antibiotic resistance of *H. pylori* is based on the analysis of cultured strains by agar diffusion or dilution or the epsilometer (E-test) (9, 15, 17). These methods are tedious and strongly dependent on the experimental conditions and subjective interpretation and hence are not always reproducible (15). DNA-based diagnostic methods may offer a rapid and reliable alternative approach for macrolide susceptibility testing. Furthermore, molecular assays can be applied directly on

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gastric biopsy specimens, without the need for in vitro culture of the bacterium (26). Therefore, we have developed a PCR-based reverse hybridization assay that permits simultaneous identification of the different mutations in the 23S rDNA (22).

The aims of the present study were to (i) assess the performance of the PCR-line probe assay (LiPA) method in a multinational study in six different laboratories, (ii) compare the results of the PCR-LiPA with results obtained by microbiological susceptibility testing, (iii) determine the prevalence of the specific 23S rDNA mutations in clarithromycin-resistant strains in different parts of the world, and (iv) investigate whether 23S rDNA mutations are related to the virulence-associated *vacA* and *cagA* genotypes.

MATERIALS AND METHODS

Multinational study. Six laboratories (in Australia, Belgium, Brazil, France, The Netherlands, and Switzerland) participated in this multicenter study. Each laboratory used its own method to culture *H. pylori* and tested for clarithromycin susceptibility by agar dilution or E-test. Each laboratory was asked to select from its strain collection approximately 50 nonduplicate and well-documented clarithromycin-susceptible and clarithromycin-resistant strains (approximately 25 of each) to be tested by PCR-LiPA. Most strains were obtained from patients after unsuccessful eradication therapy. Approval of ethics committees was obtained where necessary. All reagents and instructions used for PCR amplification of the 23S rDNA gene and reverse hybridization analysis of the PCR products by LiPA were provided by the organizing laboratory. 23S rDNA LiPA results were interpreted in each laboratory, and DNA from all strains was sent to the organizing laboratory, where the *vacA* and *cagA* genotypes were determined.

***H. pylori* cultures and isolation of DNA.** Gastric biopsy specimens were obtained from patients by upper endoscopy. From these specimens, *H. pylori* was cultured under microaerobic conditions. Genomic DNA was isolated from cultured strains by proteinase K treatment. Bacteria were harvested from plates and resuspended in phosphate-buffered saline. After centrifugation, the bacterial cell pellet was resuspended in 500 μl of a solution containing 10 mM Tris HCl (pH 8.0), 5 mM EDTA, 0.1% sodium dodecyl sulfate, and 0.1 of proteinase K per ml and incubated for at least 2 h at 55°C. Proteinase K was inactivated by incubation at 95°C for 10 min. The lysate was centrifuged at 10,000 × *g*, and the supernatant was transferred to a new tube.

Macrolide susceptibility testing. Susceptibility to macrolides was assessed by MIC testing using either agar dilution or E-test in the participating laboratories (9, 16). Each laboratory used its own MIC cutoff value to classify strains as clarithromycin susceptible or resistant.

PCR-LiPA for 23S rDNA mutations. Specific mutations in the 23S rDNA were determined by PCR-LiPA as described earlier (22). Briefly, part of the 23S rDNA was amplified by PCR, using biotinylated primers. PCR products were denatured and hybridized to specific oligonucleotide probes, which were immobilized on a nitrocellulose strip in parallel lines. This method specifically identifies A2115G, G2141A, A2142G, A2142C, A2142T, A2143G, and A2143C mutations in the 23S rDNA. Hybridization was performed under highly stringent conditions, to ensure complete specificity as described previously (22). Hybrids were detected by alkaline phosphatase-conjugated streptavidin and a substrate, resulting in a purple precipitate at the specific probe line. Hybridization results were interpreted visually.

PCR-LiPA for *vacA* and *cagA* genotyping. *vacA* (s and m region) and *cagA* genotypes were determined by PCR-LiPA as described earlier (23–25).

Statistical analyses. Statistical analyses were performed with SPSS for Windows, version 8, using the chi-square and Wilcoxon rank sum tests.

RESULTS

A total of 299 strains were studied in six different laboratories. Since the samples were tested under code, it was not possible to obtain demographic data or clinical data for the patients. First, macrolide susceptibility was tested by agar dilution ($n = 52$; 17.4%) or E-test ($n = 247$; 82.6%), and MICs were determined. Overall, among this group of selected strains, 169 (56.5%) were susceptible and 130 (43.5%) were resistant to clarithromycin.

TABLE 1. Susceptibility of *H. pylori* strains to clarithromycin and detection of 23S rDNA mutations by PCR-LiPA in six laboratories

Laboratory location	Macrolide susceptibility as determined by agar dilution or E-test	No. of strains with indicated 23S rDNA status determined by PCR-LiPA		Total
		Wild type	Mutant	
The Netherlands ($n = 51$)	Susceptible	28	2[a]	30
	Resistant	1	20	21
France ($n = 52$)	Susceptible	26		26
	Resistant	2	24	26
Australia ($n = 56$)	Susceptible	34		34
	Resistant		22	22
Belgium ($n = 51$)	Susceptible	26		26
	Resistant		25	25
Switzerland ($n = 37$)	Susceptible	19		19
	Resistant		18	18
Brazil ($n = 52$)	Susceptible	34		34
	Resistant		18	18
Total ($n = 299$)		170	129	299

[a] Contained a mixture of wild-type and A2143G mutant sequences, and the MICs were shown to vary during retesting.

The 23S rDNA was studied by PCR-LiPA, and the results are shown in Table 1. Since there were no significant differences among the laboratories, results from all strains were combined. Of the 299 strains, 170 (56.9%) contained wild-type 23S rDNA sequences, whereas mutations were detected in 129 (43.1%). As shown in Table 2, the predominant mutations among the 129 mutant strains were A2143G (44.1%) and A2142G (32.6%). The A2142C mutation was found as a single 23S rDNA genotype in two resistant strains from Belgium. In three other strains (one from France and two from Switzerland), the A2142C and A2142G mutations were both present. None of the strains contained the A2115G, G2141A, A2142T, or A2143C mutation. Uniform 23S rDNA genotypes were found in 271 (90.6%) of the strains, whereas 28 (9.4%) strains contained combinations of different 23S rDNA genotypes, suggesting either infection with multiple *H. pylori* strains or the presence of different 23S rDNA alleles in a single strain (Table 3).

There was a very strong association between the presence of 23S rDNA mutations and macrolide resistance. Overall, 167 (98.8%; 95% confidence interval, 95.8 to 99.7%) of the 169 clarithromycin-susceptible strains contained wild-type sequences, whereas 127 (96.7%; 95% confidence interval, 93.4 to 99.2%) of the 130 clarithromycin-resistant strains contained mutant 23S rDNA sequences. One of the two susceptible strains containing 23S mutations showed a mixture of the wild-type and A2143G mutant sequences.

The MICs for strains with different 23S rDNA mutations were compared (data not shown). MICs for strains containing the A2142G mutation were significantly higher than those for strains containing the A2143G mutation (Wilcoxon rank sum test, $P < 0.001$).

The *vacA* (s and m region) and *cagA* genotypes could be determined for 296 (99.0%) of the strains, and the results are shown in Table 4. There was a strong association between the *vacA* s1 genotype and the presence of *cagA* (chi-square test, $P < 0.001$). Of the 28 strains showing combinations of 23S

TABLE 2. Rate of detection of specific 23S rDNA mutations among 129 strains containing non-wild-type 23S rDNA sequences from six laboratories

Laboratory location	No. of strains with 23S rDNA mutation[a]				Total
	A2142G	A2142C	A2143G	Multiple	
The Netherlands	9		9 (1 susceptible)[b]	4 (1 susceptible)	22
France	5		13	6	24
Australia	3		9	10	22
Belgium	11	2	10	2	25
Switzerland	10		6	2 (1 susceptible)	18
Brazil	4		10	4	18
Total (%)	42 (32.6%)	2 (1.6%)	57 (44.1%)	28 (21.7%)	129

[a] The A2115G, G2141A, A2143C, and A2142T mutations were never observed.
[b] Susceptible to clarithromycin.

rDNA genotypes, only 1 (3.6%) showed multiple *vacA* genotypes. Although there were no significant associations between the *vacA* and *cagA* genotypes and the status of the 23S rDNA, a few trends were observed. First, among susceptible strains, a higher frequency of multiple *vacA* s and m genotypes was observed. Second, the subset of 99 strains containing the A2142G ($n = 42$) and A2143G ($n = 57$) mutations was analyzed. Among these, the A2143G strains more often contained multiple *vacA* s (chi-square test, $P = 0.04$) or *vacA* m (chi-square test, $P = 0.08$) genotypes than did the A2142G strains. Finally, the A2143G strains more often contained *vacA* s1- and *cagA*-positive genotypes, whereas the A2142G strains were often of the *vacA* s2- and *cagA*-negative genotype (chi-square test, $P = 0.007$ and $P = 0.04$, respectively).

DISCUSSION

Rapid and accurate detection of antibiotic resistance plays an increasingly important role in the management of *H. pylori*-infected patients. Due to the low growth rate of the bacterium, conventional susceptibility testing methods, such as agar dilution and E-test, require culture of the bacterium and are time-consuming. In contrast, molecular tools can be directly applied to gastric biopsy specimens, thereby omitting the necessity for culture (26). Since molecular methods are not dependent on bacterial density, viability, and growth rate, the results are more reliable and reproducible than results of growth-based susceptibility assays.

In the present study, the results of conventional clarithromycin susceptibility testing were compared with the results of molecular analysis by PCR-LiPA in six laboratories around the world. The strains were initially tested by agar dilution or E-test; these methods are known to be equally reliable for clarithromycin susceptibility testing (16).

The strong association between resistance to macrolides and specific mutations in the 23S rDNA was confirmed. The positive and negative predictive values of the PCR-LiPA for detection of macrolide resistance were higher than 97%, indicating the high degree of accuracy of the assay.

The PCR-LiPA method permits detection of seven distinct 23S rDNA mutations. Of these, the A2143G and A2142G mutations were the most prevalent and accounted for 98% of the macrolide-resistant strains containing single 23S rDNA genotypes. The A2142C mutation was found in only five strains.

Multiple 23S rDNA alleles were found in more than 20% of the mutant strains. Other studies also have found multiple 23S rDNA alleles (13, 14). Since *H. pylori* contains two copies of the 23S rDNA, detection of multiple 23S rDNA sequences may reflect the presence of different mutations in either copy of the gene in a single strain or may indicate the presence of multiple *H. pylori* strains. However, only 1 of the 28 strains that contained multiple 23S rDNA genotypes also showed multiple *vacA* genotypes. This may suggest that the majority of the strains containing multiple 23S rDNA genotypes were not mixed strains but were single strains containing two different mutant copies of the 23S rDNA gene, although the discriminatory power of *vacA* genotyping is obviously limited. None of the strains contained either of the A2115G and G2141A mutations, which were described by Hultén et al. (11). Also, other studies failed to identify strains with these mutations (16) among at least several hundreds of strains, implying that these particular mutants are extremely rare.

PCR-LiPA also permits specific detection of the A2142T and A2143C mutations. Strains containing these mutations have been artificially constructed and were clarithromycin resistant (3). Since both mutations appeared to be stable upon passage of *H. pylori* strains, specific probes were included in the LiPA. However, natural strains containing these mutations have not been isolated from patients so far and were also not found in the present study, suggesting that they are extremely rare or nonexistent.

All strains with discrepant results between PCR-LiPA and clarithromycin resistance testing were retested by agar dilution or E-test and PCR-LiPA. Of the initial 15 cases with discrepant results, 10 could be resolved because retesting showed a dif-

TABLE 3. Characteristics of *H. pylori* cultures containing multiple 23S rDNA genotypes

23S rDNA genotypes	No. (%) of cultures with genotypes
Wild type + A2142G	4 (14.2)
Wild type + A2143G	14 (50.0)
A2142G + A2142C	3 (10.7)
A2142G + A2143G	2 (7.1)
Wild type + A2142G + A2143G	5 (17.8)
Total	28

TABLE 4. *vacA* and *cagA* genotypes and macrolide susceptibility of *H. pylori* strains

Genotype	No. of strains with macrolide susceptibility		Total	Significance[a]
	Susceptible	Resistant		
vacA s1	101	78	179	
vacA s2	40	40	80	*vacA* s1 vs s2, $P = 0.40$
vacA s multiple	25	12	37	Single vs multiple, $P = 0.13$
vacA m1	66	46	112	
vacA m2	77	72	149	*vacA* m1 vs m2, $P = 0.29$
vacA m multiple	22	12	34	Single vs multiple, $P = 0.22$
cagA positive	118	87	205	
cagA negative	48	43	91	*cagA* negative vs *cagA* positive $P = 0.52$

[a] Chi-square test.

ferent MIC, which now was in agreement with the PCR-LiPA result (data not shown). Repeated LiPA confirmed the first results in all cases except one. In a single strain for which the MIC was >256 μg/ml, only wild-type sequences were found first, whereas retesting showed the presence of both wild-type and A2142G mutant sequences. These results show that the reproducibility of the LiPA is very high and also indicate the lack of reproducibility of conventional MIC testing.

A significant association was found between the specific 23S rDNA mutation and the MIC. The MICs for strains with the A2142G mutation were higher than those for strains with the A2143G mutation, confirming earlier observations (28). Also, the prevalences of these mutants appear to be different among strains of *H. pylori* obtained before treatment and after treatment with regimens containing clarithromycin (Y. Glupczynski et al., unpublished observations).

The A2142G and A2143G mutations were most predominant among the resistant strains. There was no significant difference among strains from different geographic origins, indicating that strains with these mutations account for the great majority of resistant strains worldwide.

Overall, 23S rDNA genotyping and susceptibility testing results for five strains remained discrepant. Two strains were clarithromycin susceptible but contained mutant 23S rDNA sequences. These findings may be due to inaccurate MIC testing, although repeated testing showed the same results. Conversely, three strains were clarithromycin resistant, but only wild-type 23S rDNA sequences were found. Again, repeated MIC testing and PCR-LiPA yielded identical results. In these strains, other mechanisms which are not related to 23S rDNA mutations may play a role (15).

Hypothetically, virulent strains of *H. pylori* will more often result in peptic ulcer disease, and patients infected by such strains will be treated. Consequently, the prevalence of macrolide-resistant strains may be epidemiologically related to the virulence of the bacterium. Considering the entire group of strains in this study, there was no significant association between the virulence-associated *vacA* and *cagA* genotypes of the *H. pylori* strains and the 23S rDNA status. However, among susceptible strains, a considerably higher frequency of multiple *vacA* s and m genotypes was observed. Clarithromycin-resistant strains are mainly obtained from patients who have been treated with antibiotics, which will often result in selection of a single resistant strain (with a single *vacA* genotype). In contrast, clarithromycin-susceptible strains are mainly obtained from patients who have not been treated and have not been subjected to the antibiotic selection pressure. Therefore, multiple strains will be less prevalent among clarithromycin-resistant strains.

In a subgroup of 99 strains (containing either the A2142G or the A2143G mutation), there were significant differences between A2143G strains and A2142G strains with respect to the frequency of *vacA* and *cagA* genotypes. This may indicate that there is a weak clonal relationship between virulence-associated and 23S rRNA genes, although the mechanism remains unclear. This weak relationship is a further indication that the mechanism mentioned above may influence the selection of resistant and maybe more-virulent strains from a mixed infection present before the onset of therapy, probably resulting in a weak clonal relationship between virulence-associated and 23S rRNA genes. However, a bias in the selection of the strains in the present study population cannot be excluded, as they all represent strains obtained from patients who underwent gastroscopy for upper abdominal complaints. Therefore, this selection bias might result in the overrepresentation of certain virulence-associated genotypes in the isolates tested.

In conclusion, macrolide resistance of *H. pylori* can be effectively determined by molecular analysis of 23S rDNA mutations. PCR-LiPA offers a reliable molecular tool for rapid and accurate identification of macrolide-resistant *H. pylori* strains. The use of such tools, especially when used directly with gastric biopsy specimens, may facilitate the choice of effective antibiotic therapies for *H. pylori* infection and prevent further increase of the prevalence of antibiotic resistance.

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