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Evaluation of Clarithromycin Resistance and *cagA* and *vacA* Genotyping of *Helicobacter pylori* Strains from the West of Ireland Using Line Probe Assays

KIERAN A. RYAN,[1,2] LEEN-JAN VAN DOORN,[3] ANTHONY P. MORAN,[2] MAURA GLENNON[1] TERRY SMITH,[1] AND MAJELLA MAHER[1]*

National Diagnostics Centre, BioResearch Ireland,[1] and Department of Microbiology,[2] National University of Ireland, Galway, Ireland, and Delft Diagnostics Laboratory, Delft, The Netherlands[3]

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The prevalence of clarithromycin resistance-associated mutations, the cytotoxin-associated gene (*cagA*), and the various vacuolating cytotoxin (*vacA*) genotypes was determined in 50 gastric biopsy specimens from *Helicobacter pylori*-infected patients, using line probe assays. The clarithromycin resistance-associated mutation A2143G was detected in *H. pylori* strains from 26% of the specimens, which suggested that the high rate of *H. pylori* treatment failure in Ireland may be partly attributable to the presence of these mutations. All strains examined carried the *vacA* s1 genotype, and 76% were *cagA* positive. Of these 50 specimens, 13 (26%) carried *H. pylori* strains with *vacA* midregion genotype m1, 29 (58%) carried strains that were m2, 1 (2%) was infected by a strain that was positive for both m1 and m2, and 7 (14%) carried strains that could not be typed.

Helicobacter pylori is a gram-negative spiral bacterium that colonizes and persists in human gastric mucosa. *H. pylori* infects more than half of the world's population, and it is implicated as a causative agent of gastritis, peptic ulcer disease, carcinoma, and lymphoma (5).

Treatment of *H. pylori* infection has been the subject of a large number of clinical trials in recent years. The current treatment of choice consists of a triple or quadruple regimen that includes antibiotics (usually metronidazole, clarithromycin, amoxycillin, or tetracycline) and a proton pump inhibitor such as omeprazole, lansoprazole, or pantoprazole (3, 9). To date, there is no single standardized treatment regimen, and treatment failure is still reported to be highly frequent (3, 21). Many factors have been implicated as causes of treatment failure, including ineffective penetration of antibiotics into the gastric mucosa, antibiotic inactivation by low stomach pH, lack of patient compliance, and the emergence of acquired resistance to antibiotics by *H. pylori* (1, 19).

The resistance of *H. pylori* to clarithromycin has been shown to be due to point mutations at the peptidyltransferase region of domain V of the 23S rRNA (15, 20). These mutations prevent binding of the macrolide and thus lead to resistance. Two copies of the 23S rRNA gene are present in *H. pylori*, and to date at least five distinct mutations have been described, including A→G transitions at positions 2142 (A2142G) and 2143 (A2143G) (20), an A→C transversion at position 2142 (A2142C) (12), and G→A transitions at positions 2115 (G2115A) and 2141 (G2141A) (6). Although resistance to metronidazole is high, occurring in an average of 27% of cases in Europe and up to 90% of cases in Africa (10), resistance to clarithromycin is not as prevalent, with frequencies that range from 1% in Norway to 10% in France (10, 11).

* Corresponding author. Mailing address: DNA Diagnostics Laboratory, The National Diagnostics Centre, BioResearch Ireland, National University of Ireland, Galway, Ireland. Phone: 35391-586559. Fax: 35391-586570. E-mail: Majella.Maher@nuigalway.ie.

The primary aim of this study was to investigate the presence of mutations that confer resistance to clarithromycin in DNA samples extracted directly from stomach biopsy specimens, using a previously validated molecular test (16). This work was prompted by reports of very high rates of *H. pylori* treatment failure in Ireland (21). Furthermore, the study aimed to genotype the *vacA* gene and detect the *cagA* gene in these strains from the west of Ireland, since a study previously undertaken by our laboratory found a high frequency of both the *cagA*-positive and the *vacA* signal region genotype s1 strains among *H. pylori* isolates in Ireland (13).

A total of 70 antral biopsy specimens were collected at the open endoscopy unit, University College Hospital, Galway, Ireland, from patients displaying a range of pathologies: 34 with gastritis, 18 with ulcers, 12 with reflux esophagitis, 4 with nonulcer dyspepsia, and 2 who were asymptomatic. The specimens were stored immediately at −80°C until processing. After the specimens were thawed, total DNA was extracted using the Puregene genomic DNA isolation kit (Gentra Systems, Minneapolis, Minn.). The DNA isolation protocol for frozen solid tissue was followed according to the manufacturer's instructions. DNA was suspended in 100 μl of nuclease-free H_2O and stored at −20°C.

PCR was carried out for each sample in a volume of 100 μl using previously described primers specific for the *ureB* gene (4). Analysis of PCR products was by 2% agarose gel electrophoresis. To confirm *H. pylori*-positive samples, Southern blot hybridization analysis was performed with a digoxigenin (DIG)-labeled DNA probe, UREPRO (5′-GGCAAAGT GGCTGACTTGGT-3′), which is a *ureB*-specific probe designed in our laboratory to anneal to a 20-bp sequence in the *ureB* PCR product (nucleotides 2004 to 2024). The probe was labeled with DIG using the DIG oligonucleotide 3′-end labeling kit (Roche Diagnostics, Mannheim, Germany), and detection procedures were as described in the DIG luminescent kit for nucleic acids (Roche Diagnostics). Results were recorded

after exposure to autoradiographic film for 1 h. Samples were scored as *H. pylori* positive if a *ureB* PCR product was detected following Southern blot hybridization analysis with the URE-PRO probe.

A fragment of the 23S rRNA gene was amplified by PCR as previously described (16). Biotinylated PCR products were analyzed by reverse hybridization by a line probe assay (LiPA). The 23S rRNA LiPA contains probes specific for seven 23S rRNA mutations along with two probes specific for wild-type strains; the probes are immobilized in parallel lines on a nitrocellulose membrane strip (16). Following hybridization and stringency washes, PCR-DNA probe hybrids were detected by the addition of conjugate (streptavidin-alkaline phosphatase) and substrate (4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate). Results were visually interpreted. To further validate the method, simultaneous detection was performed with set of previously described controls (16).

The presence of *cagA* and the subtypes of the *vacA* signal and midregions was determined by multiplex PCR followed by reverse hybridization by a second LiPA, as described previously (17). The *vacA* signal region types s1a, s1b, s1c, and s2 and midregion types m1, m2a, and m2b can be detected with this LiPA (17). The same hybridization and detection methods described above were employed. To confirm the *vacA* midregion type in some samples, a standard PCR and gel electrophoresis method was employed using previously described primers (2).

Of the 70 biopsy specimens collected, 50 tested positive for *H. pylori*, using the *ureB* DNA hybridization method as described. These 50 DNA samples were then evaluated in the 23S rRNA and *cagA*-*vacA* LiPAs. Thirteen (26%) of the DNA samples tested positive for the A2143G mutation by the 23S rRNA LiPA. Hybridization of control PCR products for designated mutations had the expected results. No other mutations were observed, and all other DNA samples (74%) displayed the wild-type genotype.

Table 1 shows the number of patients from each pathology group with *H. pylori* infections that had the same combination of *vacA* genotype, *cagA* status, and 23S rRNA genotype. All DNA samples except one, which was s1b, were *vacA* signal region type s1a. The s1c genotype was not detected in our group of strains. Both m1 and m2 *vacA* midregion genotypes were observed. The m2b genotype was not detected. The *vacA* midregion genotype of 11 DNA samples could not be determined using the LiPA method, due to the lack of a visible signal on the strips. In four cases, results were obtained using the PCR method of Atherton et al. (2), but the *vacA* midregion genotype of the remaining samples could not be determined. Thirty-eight of the 50 DNA samples (76%) were *cagA* positive.

We observed clarithromycin resistance-associated mutations in 26% of the *H. pylori* strains in biopsy specimens from the west of Ireland. This prevalence is high compared to the resistance found in other European countries (10, 11). A previous combined study that included 60 *H. pylori* strains from Ireland and the United Kingdom has reported a 5% frequency of resistance to clarithromycin (11). In our study, only the A2143G 23S rRNA mutation was observed. This mutation predominates in *H. pylori* strains from Europe (1, 14) and also in those from Japan (7). It has been shown that resistance to clarithromycin is a strong predictor of *H. pylori* eradication

TABLE 1. Characteristics of 50 *H. pylori*-positive biopsy specimens from patients in Ireland

H. pylori characteristics			No. of patients in each pathology group			
vacA genotype	*cagA* status[d]	23S rRNA genotype[b]	GAS	ULC	RE	NUD
s1a, m1	*cagA* pos.	sen	3	2	0	0
s1a, m1	*cagA* neg.	sen	0	1	1	0
s1a, m1	*cagA* pos.	res	2	0	3	0
s1a, m1	*cagA* neg.	res	0	0	1	0
s1a, m2a	*cagA* pos.	sen	6	7	2	1
s1a, m2a	*cagA* neg.	sen	7	2	0	0
s1a, m2a	*cagA* pos.	res	2	1	0	0
s1a, m2a	*cagA* neg.	res	0	0	0	0
s1b, m2a	*cagA* neg.	sen	1	0	0	0
s1a, m1+m2a	*cagA* pos.	sen	1	0	0	0
s1a, ND[c]	*cagA* pos.	sen	1	2	1	0
s1a, ND	*cagA* pos.	res	2	0	0	1

[a] GAS, gastritis; ULC, ulcer; RE, reflux esophagitis; NUD, nonulcer dyspepsia.
[b] sen, clarithromycin-sensitive genotype; res, clarithromycin-resistant genotype.
[c] ND, not determined.
[d] pos., positive; neg., negative.

treatment failure (1). We suggest that the high rate of resistance-associated mutations we observed might be a contributing factor to the unusually high rate (48%) of treatment failures in Ireland (21).

As observed in our previous study (13), only the s1 *vacA* signal region genotype was present in the *H. pylori* strains from these Irish patients. Thus, s1 strains were present in all of the patient pathologies irrespective of disease severity. Both *vacA* midregion genotypes m1 and m2 were observed, but there was no association between m1 strains and more severe patient pathologies. It would appear, as previously suggested (13), that in the Irish study, *vacA* genotypes were not useful predictors of disease severity. The occurrence of only one 23S rRNA gene mutation and only one *vacA* signal region genotype provided further evidence suggesting that the *H. pylori* strains found in Ireland are genetically very homogeneous compared to those of other geographic locations (18).

Using the LiPA method, the *vacA* midregion genotype of 11 strains could not be determined. The midregion PCR is the least sensitive part of the multiplex system (L. J. van Doorn, personal communication). PCR amplification of the midregion in this multiplex PCR might have been successful for the DNA extracted from these biopsy specimens had the DNA been resuspended in a smaller volume at the end of the DNA extraction process. Lack of a *vacA* midregion signal with the LiPA may also have been due to the presence of DNA polymerase inhibitors in the DNA sample left over from the extraction procedure, which could reduce the overall sensitivity of the multiplex PCR amplification. Each of the 11 strains that could not be typed for the *vacA* midregion using the multiplex PCR/LiPA returned a positive and interpretable signal for *cagA* and the *vacA* signal region. These signals indicated that the LiPA probes detected PCR products amplified in the multiplex and that the failure of the LiPA to return an interpretable result for the midregion was most likely related to the failure to achieve PCR amplification of the required sensitivity. Therefore, it appears that the choice of extraction method and

the final DNA concentration are crucial to obtaining optimal results when carrying out this method directly on gastric biopsy samples. Gastric biopsy specimens of adequate size also should be used. However, although a specific midregion PCR (2) did yield positive results for four strains (all of which were m2), a positive PCR signal was not obtained for the remaining seven DNA samples. It is possible that these strains could not be typed due to the presence of *vacA* midregion sequence variation; however, further investigation would be required to establish this as a cause.

Seventy-six percent of strains in this study were *cagA* positive. In a previous study, we reported that 93% of strains were *cagA* positive (13). However, in the earlier study the strains used were not chosen randomly and included a large percentage of strains from patients with intestinal metaplasia (a gastric cancer precursor state). We found that the rate of *cagA*-positive strains in Ireland, as reported here, is similar to that found in other European countries (17, 20). When patients were divided into ulcer ($n = 15$) and nonulcer ($n = 35$) groups, there was a higher but statistically insignificant ($0.5 < P < 0.75$) percentage of *cagA*-negative strains in the nonulcer group (35%) than in the ulcer group (25%). It seems that, as previously suggested (8, 13), *cagA* predominates in patients with more severe pathologies but is not a predictor of eventual disease outcome in the Irish population.

In Ireland, *H. pylori* eradication therapy is usually performed with a proton pump inhibitor and a combination of two antibiotics, chosen from clarithromycin, metronidazole, and amoxycillin. At present, the rate of failure of *H. pylori* eradication therapy is 48% in this country (21). We believe that this high failure rate can be attributed, at least in part, to the high percentage of strains carrying the clarithromycin-resistant genotype, as reported here. The increasing resistance of *H. pylori* to antibiotics highlights the importance not only of the continuing the search for new therapeutic measures but of ensuring better patient compliance with current regimens through improved education strategies.

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