High Prevalence of Clarithromycin-Resistant Helicobacter pylori Strains and Risk Factors Associated with Resistance in Madrid, Spain

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Clarithromycin is one of the antibiotics used for the treatment of Helicobacter pylori infections, and clarithromycin resistance is the most important factor when it comes to predicting eradication failure. The present study analyzed H. pylori isolates for the presence of 23S rRNA gene mutations and determined the risk factors associated with resistance among H. pylori isolates collected in Madrid, Spain, in 2008. We studied 118 H. pylori strains isolated from the same number of patients. A total of 76.3% of the patients were born in Spain, 52.7% were children, 20.3% had previously been treated, and 66.1% were female. Clarithromycin resistance was determined by Etest. H. pylori strains were considered resistant if the MIC was ≥1 mg/liter. DNA extraction was carried out by use of the NucliSens easyMAG platform with NucliSens magnetic extraction reagents (bioMérieux). The DNA sequences of the 23S rRNA genes of clarithromycin-resistant and -sensitive strains were determined to identify specific point mutations. The vacA genotype and cagA status were determined by PCR. We found that 42 (35.6%) strains were resistant to clarithromycin by Etest. Etest results were confirmed by detection of the presence of point mutations in 34 (88.1%) of these strains. Eight H. pylori strains were resistant to clarithromycin by Etest but did not have a point mutation in the 23S RNA gene. Mutation at A2143G was found in 85.3% of the strains, mutation at A2142G in 8.8%, and mutation at T2182C in 5.9%. Dual mutations were found in 8.8% of the strains. H. pylori clarithromycin-resistant strains were strongly associated with pediatric patients, with patients born in Spain, and with patients who had previously been treated (P ≤ 0.02). In addition, H. pylori strains resistant to clarithromycin more frequently presented the vacA s2/m2 genotype and were more likely to be cagA negative than susceptible strains (39.1% and 11.2%, respectively; P value < 0.001). We concluded that, in the present study, H. pylori clarithromycin-resistant strains are more frequently found in children, in patients mostly born in Spain, and in individuals who were previously treated for H. pylori infection and that these individuals are more likely colonized with a less virulent H. pylori strain.

**Helicobacter pylori** is a microaerobic, Gram-negative spiral bacterium that colonizes the human stomach and is found in more than half of the world’s population (32). Infections with *H. pylori* are closely associated with chronic gastritis, peptic ulcer disease, and the development of gastric cancer (8, 32).

All consensus guidelines recommend eradication of *H. pylori* for patients with symptoms (9, 28). Standard therapy combines a proton pump inhibitor (PPI) or ranitidine bismuth citrate and two antibiotics, chosen from among amoxicillin, clarithromycin, and metronidazole (24, 25). However, this therapy has been questioned because of the increased eradication failure rates. Many factors have been implicated as causes of treatment failure, including ineffective penetration of antibiotics into the gastric mucosa, antibiotic inactivation by the low stomach pH, a lack of patient compliance, and the emergence of acquired resistance to antibiotics by *H. pylori* (26, 27).

In many cases, clarithromycin is the key component of these combination therapies. However, resistance to clarithromycin has become one of the major reasons for treatment failure (13). The prevalence of *H. pylori* resistance to clarithromycin varies among different countries, such as 10.6% to 25% in North America, 16% in Japan, and 1.7% to 23.4% in Europe (14, 19, 21). Overall, resistance to clarithromycin has been detected more in patients living in the south than in those living in the north of Europe (21). Fewer studies have focused on the prevalence of clarithromycin resistance among *H. pylori* strains from children compared with that among strains from the adult population. These studies will be useful for estimating the rate of clarithromycin-resistant *H. pylori* isolates among children and adults in Spain in the future (1, 23).

Clarithromycin acts by binding to the peptidyltransferase region of 23S rRNA and inhibits protein synthesis (36). The resistance to clarithromycin in *H. pylori* has been shown to be due to point mutations in the peptidyltransferase region of domain V of the 23S rRNA. Two copies of the 23S rRNA gene are present in *H. pylori*, and the most common mutation is an A-to-G transition at position 2143 (A2143G) (13, 36), but several point mutations, at positions A2142G, A2144G, and T2182C, have been described. Recent reports have indicated that other mutations, such as A2115G, G2141A, C2147G, T2190C, C2195T, A2223G, and C2694A, might also be associated with clarithromycin resistance (20, 31). Other mechanisms of resistance, such as methylase production, the actions of macrolide-inactivating enzymes, and active efflux, have been described in several bacteria. Active efflux has also recently been described in *H. pylori* (22).

Since the worldwide increase in the rate of clarithromycin resistance represents a problem of relevance, some studies...
have been performed in order to identify its relationship with bacterial genetic factors (12, 35, 38).

Two genes (cytotoxin-associated gene A [cagA] and vacuolation-associated gene A [vacA]) have been identified to be the main virulence factors. cagA is located in the cag pathogenicity island (PAI), which encodes a type IV secretion system, and the presence of cagA is closely associated with more severe gastric diseases (2, 15, 34). The VacA toxin induces vacuole formation in the host cells. There is considerable variation in vacuolation activity among H. pylori strains, primarily due to differences in the vacA gene structure in the signal region (s1 and s2) and the middle region (m1 and m2). vacA s1/m1 and s1/m2 produce high and moderate levels of VacA toxin, respectively, whereas s2/m2 produces little or no toxin (11). A strong association between clarithromycin susceptibility and these virulence factors has been reported (12, 38).

The focus of the present study was to evaluate the distribution of clarithromycin-resistant H. pylori strains and their association with genotypic markers, such as the cagA gene and allelic variants of the vac gene. We also examined the distribution of H. pylori clarithromycin resistance in relation to the patient’s age, place of birth, and history of treatment. Our main goal is to determine potential host and bacterial factors that may help in predicting resistance to clarithromycin among H. pylori isolates.

MATERIALS AND METHODS

Patients. A total of 118 H. pylori clinical strains were studied, including 61 obtained from children who were <18 years of age (27 males, 34 females) and 57 obtained from adults ≥18 years of age (13 males, 44 females). The strains were isolated from gastric biopsy specimens obtained during upper gastroduodenal endoscopy from two children's hospitals (Hospital Infantil Universitario Niño Jesús and Hospital Universitario Doce de Octubre, Madrid, Spain) and an adult hospital (Hospital Universitario de la Princesa, Madrid) from May 2006 to December 2008. Most of the patients (76.3%) were born in Spain, and 20.3% of them had previously been treated for H. pylori infections. However, the patients had not received PPIs or antibiotics for at least 2 weeks prior to the endoscopy and before initiation of any new therapy. Each patient or the patient's parents signed an informed-consent form that was previously approved by the ethics committee of each hospital.

Clinical isolates. Samples for culture were placed in sterile saline solution for transport. Biopsy specimens were received at the Department of Microbiology (Hospital Universitario de la Princesa) and processed in less than 3 h from the time that the biopsy specimens were obtained. Tissue was streaked onto both nonselective medium (Columbia agar with 5% sheep blood; BioMérieux, Marcy l'Etoile, France) and selective medium (Pylori agar; BioMérieux) and incubated for 10 days at 37°C in a microaerobic atmosphere (5% O2, 10% CO2, 85% N2) that was recovered in 55 μl of silica. This was followed by automatic magnetic separation. Nucleic acid extraction. The total bacterial genomic DNA of the 118 isolates was extracted by using a NucliSens easyMag instrument (BioMérieux), according to the manufacturer's instructions. Briefly, the bacterial cell suspension was pretreated with lysis buffer. The lysed sample was transferred to a plastic vessel with 550 μl of silica. This was followed by automatic magnetic separation. Nucleic acid was recovered in 55 μl elution buffer. The DNA concentration was determined on a NanoDrop instrument (NanoDrop Technologies, Wilmington, DE).

Determination of clarithromycin resistance by phenotypic methods. We prepared 48-h cultures of H. pylori that were suspended in sterile saline and adjusted to a density equal to McFarland turbidity standard of 3, as recommended by CLSI (10). The bacterial suspensions were spread onto Mueller-Hinton blood agar plates (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD) with sterile cotton swabs. The MIC of each isolate was determined by the Epsilometer test (Etest; AB Biodisk, Solna, Sweden) on Mueller-Hinton sheep blood agar plates (BBL). Plates with strips containing clarithromycin were incubated for 72 h under microaerophilic conditions. The MIC was considered the lowest concentration of drug which inhibited visible growth and was read as the intercept of the elliptical zone of inhibition with the graded strip for the Etest. On the basis of CLSI recommendations (10), strains were resistant if the MIC was ≥1 mg/liter and intermediate if the MIC was 0.5–1 mg/liter. Strains with MICs below those thresholds were considered susceptible.

Determination of clarithromycin resistance by genotypic methods. To detect specific mutations for clarithromycin resistance in the 23S rRNA gene, PCR methods were carried out. Primers were designed from the 23S rRNA gene of reference strain of H. pylori 26695. The primers used included 5'-CCACAGCG ATGTTGCTCAG (sense, positions 1891 to 1911) and 5'-CCCTCAAGAG CCAAAGGCC (antisense, positions 2210 to 2220). The numbers represent the location of the 23S rRNA gene of H. pylori 26695. Amplification was carried out in a thermal cycler (MBS O. S; Thermo Hybaid). PCR cycling conditions consisted of 35 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 30 s of extension at 72°C. PCR products were purified by using a QIAquick PCR purification kit (Qiagen, Valencia, CA). Sequencing reactions were performed by the Macrogen USA Company (Baltimore, MD) and were carried out in a BigDye Terminator cycle sequencing kit. Computer sequence alignments were performed with the Chardant program (European Bioinformatics Institute [http://www.ebi.ac.uk/chardant/]). Sequence comparisons were carried out with the Genedoc program (www.nrbc.gov.uk/genedoc/index.htm).

An extra PCR amplification was performed on eight strains that showed discrepancies between phenotypic and genotypic methods. We designed primers derived from the 23S rRNA sequence of the H. pylori 26695 reference strain (GenBank accession number U27270; sense primer, 5'-TCCCTCTCTAACTA CGGGA [5'-CTGGCATGAAATA]; antisense primer, 5'-CTGCA-CCACGTGGCGG; AACCAGAG [postions 2731 to 2752]). PCR products were sequenced to identify new mutations or mutations different from those published previously (20, 31).

Assessment of cagA and vacA status and gene amplification. The purified DNA from 118 H. pylori strains was subjected to PCR for detection of the H. pylori cagA gene, using the CagA primers described by Panayotopoulou et al. (29), which amplified a region of between 300 and 700 bp. The sense primer (s) region of the vacA gene was amplified using the primers described by Atherton et al. (6), which evaluated the region encoding the S1 region of the gene. The m-region (m) was amplified using the primers described by G. Perez-Perez et al. (unpublished data; sense primer, 5'-CAATAGCATCATTGACAG; antisense primer, 5'-TCAAGTTTTGTGTATTGAC). Four different PCR products were obtained: s1 (176 bp), s2 (200 bp), m1 (290 bp), and m2 (350 bp).

Statistical analysis. Differences between groups were statistically evaluated by using the chi-square test with Yates's correction. Differences were considered significant at the 5% probability level. Statistical analysis was performed using specific software (Epi Info; www.cdc.gov/epiinfo).

RESULTS

Clarithromycin resistance. The assessment of clarithromycin susceptibility by Etest showed that 75 patients (63.6%) were infected with H. pylori-susceptible strains and 42 (35.6%) with H. pylori-resistant strains. To confirm the high prevalence of resistance to clarithromycin, we determined the correlation between the results of Etest and sequence analysis of the 23S rRNA gene. There was a strong association between the presence of 23S rRNA gene mutations and macrolide resistance. Overall, 34 of the 42 (81%) clarithromycin-resistant strains contained at least one point mutation in their 23S rRNA sequences. We also tested 20 clarithromycin-susceptible H. pylori strains and found no point mutation in any of the 23S rRNA sequences. Thus, using classical and molecular methods, we demonstrated that 27.5% of H. pylori strains in Madrid were clarithromycin resistant. There was a discrepancy between the two methods for eight (19%) strains; the strains were resistant to clarithromycin by Etest, but no point mutation was identified in the sequence of the 23S rRNA gene. In addition, for one strain it was not possible to perform the Etest, and the
We found a relationship between clarithromycin susceptibility and \( H. \) pylori isolates from previously treated patients were more often resistant to clarithromycin than strains isolated from patients born outside Spain (33.7\% and 14.3\%, respectively; \( P = 0.05 \)).

Analysis of a possible association between \( H. \) pylori clarithromycin resistance and the gender of the patient showed that females were more often infected by resistant isolates in almost identical proportions (30.8\% and 25\%, respectively).

### DISCUSSION

\( H. \) pylori eradication usually entails the use of a proton pump inhibitor or bismuth salts in combination with two antibiotics. Resistance of \( H. \) pylori to antimicrobials agents is the main cause of treatment failure. Clarithromycin is recognized as the key antibiotic for \( H. \) pylori treatment since it has the most powerful bactericidal effect in vitro compared to the effects of the other available antibacterial agents. Unfortunately, the level of primary clarithromycin resistance is increasing worldwide, and the level of resistance to clarithromycin varies between different geographical regions. The severity of gastric inflammation, the dosage of proton pump inhibitor, and the pathology (peptic ulcer disease versus non-peptic ulcer disease) also affect the outcome of therapy (33, 38).

The first relevant finding of the present study is that the rate of \( H. \) pylori resistance to clarithromycin is high, occurring in nearly 30\% of the strains isolated in our geographic area. Spain has one of the highest levels of clarithromycin resistance reported in Europe (1, 21). Such an observation is important in order to treat patients. Patients born in Spain were more often colonized with resistant strains than immigrant patients born outside Spain. The majority of these patients are from South America or Eastern European countries, where the level of resistance to clarithromycin is lower than that in the south of Europe, and for this reason they may be infected with less resistant strains (4, 7, 16, 37).

Clarithromycin resistance in \( H. \) pylori mainly results from point mutations in the peptidyltransferase loop region of the 23S rRNA gene. In this study, the most frequent point mutation found was at position A2143G in 85\% of our strains. This mutation predominates in \( H. \) pylori strains isolated from Europe and also from Japan (13, 19). The strong association between resistance to macrolides and specific mutations in the

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**TABLE 1. vacA genotype distribution among 118 cagA-positive and cagA-negative \( H. \) pylori strains**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. (%) of strains</th>
<th>Total</th>
<th>cagA positive (n = 44)</th>
<th>cagA negative (n = 74)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>s1/m1 or s1/m2</td>
<td>38</td>
<td>35 (79.6)</td>
<td>3 (4.0)</td>
<td></td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>s2/m2</td>
<td>72</td>
<td>3 (6.8)</td>
<td>69 (93.2)</td>
<td></td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>s2/m1</td>
<td>4</td>
<td>3 (6.8)</td>
<td>1 (1.4)</td>
<td></td>
<td>NSa</td>
</tr>
<tr>
<td>Mix</td>
<td>4</td>
<td>3 (6.8)</td>
<td>1 (1.4)</td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

a NS, not significant.

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**TABLE 2. Association between clarithromycin susceptibility and cagA status among 117 \( H. \) pylori strains isolated from Spain**

<table>
<thead>
<tr>
<th>cagA status</th>
<th>No. (%) of strains</th>
<th>Total (n = 117)</th>
<th>Clarithromycin susceptible (n = 75)</th>
<th>Clarithromycin resistant (n = 34)</th>
<th>Discrepant (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>44</td>
<td>36 (81.8)</td>
<td>5 (11.4)</td>
<td>3 (6.8)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>73</td>
<td>39 (53.4)</td>
<td>29 (39.7)</td>
<td>5 (6.8)</td>
<td></td>
</tr>
</tbody>
</table>

a For the values of 81.8\% versus 53.4\%, \( P < 0.001 \) (chi-square test).
23S rRNA gene was confirmed in 81% of the cases. However, there were discrepancies between the results of sequence analysis of the 23S rRNA gene and clarithromycin resistance tested by Etest for eight strains. All eight strains were resistant by Etest but did not present any of the point mutations in the 23S rRNA gene sequence described above. PCR amplification was performed in order to amplify the conserved 695-bp region of H. pylori 23S rRNA to look for a point of mutation in these strains.

Recent reports indicated that other mutations, such as A2115G, G2141A, C2147G, T2190C, C2195T, A2223G, and C2694A, might also be associated with clarithromycin resistance (20, 31). For this reason, we performed a second PCR that amplified a larger region of the 23S rRNA gene in order to look for these point mutations, but we did not find any of them. On the basis of these results, we suggested that in these strains other mechanisms which are not related to the 23S rRNA gene sequence, such as the presence of an efflux pump, may play a role in resistance to clarithromycin, as has been described by other authors (22).

An expected finding of this study was the relationship between clarithromycin resistance and age. The rate of clarithromycin resistance was significantly higher in children than adults, and this type of association has been reported previously (30). This disparity in resistance rates seems to be correlated to the national level of macrolide consumption, since a crossover resistance mechanism among different types of macrolides develops rapidly. New macrolides were marketed in Spain at the beginning of the 1990, and clarithromycin began to be marketed in 1991. Children have had more exposure to macrolides, and nowadays, respiratory infections in young children are very frequently treated with this group of antibiotics (1, 23).

The antibiotic resistance of H. pylori is the most important reason for failure of its eradication. Patients who received treatment against H. pylori were colonized with a clarithromycin-resistant strain more often than patients who did not receive treatment. This antibiotic is the one most frequently included in the standard triple therapies for H. pylori eradication, and treatment failure was most often explained because the original strains were resistant to clarithromycin in many cases (13). To avoid treatment failure and the consequent development of secondary resistance, it is important to choose the most appropriate first-line treatment regimen. This choice should be made on the basis of knowledge of the antimicrobial resistance peculiar to a given geographical area.

The cagA and vacA genotype markers are widely used to characterize H. pylori virulence in relation to disease severity. Several epidemiological studies have shown geographical variations in its virulence factors, such as the cagA locus and the mosaic combination of the vacA gene alleles. The assessment of vacA gene mosaicism found all possible combinations; the s2/m1 mosaicism is rare, but has been reported before (33). s2/m2 was the vacA mosaicism detected the most often, and this combination was detected more frequently in cagA-negative strains than in cagA-positive strains. The presence of cagA is strongly associated with the presence of the vacA s1 allele. The prevalence of cagA and the s1 and m1 vacA alleles found in our series was similar to that observed in other studies in our region (3). This distribution differs from that observed in other countries, such as the United States, where s1/m1 and s2/m2 are equally prevalent, and Germany and the United Kingdom, where s1/m1 and s1/m2 are the most frequent combinations, respectively (11).

Our results indicate a difference in sensitivity to clarithromycin between the H. pylori genotypes. The present study indicated that s2/m2 strains, which are mostly cagA negative, seem to be more resistant to clarithromycin than strains with the s1/m1 and s1/m2 mosaic combinations, which are mostly cagA positive. It suggests that these genetic patterns could provide a selective advantage during bacterial replication. Isolates that are cagA positive, a genotype which is thought to be more virulent, may damage the gastric epithelium more, and it is conceivable that antibiotics can reach higher concentrations in inflamed mucosa. The presence of s2/m2 strains that induce less inflammation in the host gastric epithelia may be a factor contributing to reduced antibiotic delivery and could hamper the eradication of H. pylori. Another possible explanation, according to the results of other studies, is that antibiotic activity interferes with the metabolism of a dividing cell, and cagA-positive strains may proliferate faster than cagA-negative ones and would therefore be more susceptible to antibiotics (38).

In conclusion, the prevalence of H. pylori clarithromycin resistance is high in our area. Being born in Spain, being a child previously treated for H. pylori infection, or being colonized with a cagA-negative strain was correlated with the presence of a clarithromycin-resistant strain.

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