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

Sonia Agudo,^{1*} Guillermo Pérez-Pérez,² Teresa Alarcón,¹ and Manuel López-Brea¹

Hospital de la Princesa, Universidad Autónoma de Madrid, Madrid, Spain,¹ and Departments of Medicine and Microbiology, NYU School of Medicine, New York, New York²

Received 22 January 2010/Returned for modification 30 March 2010/Accepted 19 July 2010

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 rRNA 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 ($P \leq 0.02$). 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

* Corresponding author. Mailing address: Department of Microbiology, Hospital Universitario de la Princesa, Diego de León, 62, 28006 Madrid, Spain. Phone and fax: 0034915202317. E-mail: soniaagu@hotmail.com.

[▽] Published ahead of print on 28 July 2010.

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 2008 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) which was produced by a microaerobic generator envelope (GasPak EZ gas generator; Becton Dickinson and Company, MD). Isolates were presumptively identified as *H. pylori* on the basis of colony morphology, (gray, small, and translucent colonies after 3 to 4 days of incubation on blood agar), positive biochemical reactions (cytochrome oxidase, catalase, and urease), and Gram staining (curved rod), as recommended elsewhere (5, 17, 18).

DNA 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 pre-treated 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

Epsilonometer 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 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 ATGTGGTCTCAG (sense, positions 1891 to 1911) and 5'-CTCCATAAGAG CCAAAGCCC (antisense, positions 2200 to 2220). The numbers represent the positions in the 23S rRNA gene of *H. pylori* 26695. Amplification was carried out in a thermal cycler (MBS O.5 S; Thermo Hybad). 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. The PCR products were purified 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 Clustal program (European Bioinformatics Institute [http://www.ebi.ac.uk/clustalw/]), and sequence comparisons were carried out with the Genedoc program (www.nrbsc.org/gfx/genedoc/index.html).

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'-TCCTCTCGTACTA GGGA [positions 2057 to 2074]; antisense primer, 5'-CTGCATGAATGGCGT AACGAG [positions 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 360 and 700 bp. The signal peptide (*s*) region of the *vacA* gene was amplified using the primers described by Atherton et al. (6), which evaluated the region encoding the *s* region of the gene. The midregion (*m*) was amplified using the primers described by G. Perez-Perez et al. (unpublished data; sense primer, 5'-CAATCTGTCCAATCAAGCGAG; antisense primer, 5'-TGAGTTGTTTGATATTGAC). 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 Yate'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 27.2% 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

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

Genotype	No. (%) of strains			<i>P</i>
	Total	<i>cagA</i> positive (<i>n</i> = 44)	<i>cagA</i> negative (<i>n</i> = 74)	
<i>s1/m1</i> or <i>s1/m2</i>	38	35 (79.6)	3 (4.0)	<i>P</i> < 0.0001
<i>s2/m2</i>	72	3 (6.8)	69 (93.2)	<i>P</i> < 0.0001
<i>s2/m1</i>	4	3 (6.8)	1 (1.4)	NS ^a
Mix	4	3 (6.8)	1 (1.4)	NS

^a NS, not significant.

results for that strain were excluded from further statistical analysis.

The predominant mutations among the 34 *H. pylori* clarithromycin-resistant strains were at A2143G in 26 cases (76.6%), A2142G in 2 cases (5.8%), and T2182C in 2 cases (5.8%), while double point mutations of A2143G plus T2182C were found in 3 cases and a double mutation of A2142G plus T2182C was found in 1 case. In addition, there were no differences in the MIC values among isolates with a point mutation in A2143G, A2142G, or T2182C.

Clarithromycin resistance relationship with *cagA* gene and *vacA* mosaicism distribution. The *cagA* and *vacA* (*s* and *m* region) genotypes of the 118 *H. pylori* strains were assessed. The *cagA* gene was detected in 44 strains (37.3%). In the case of the *vacA* gene, we found that 38 (32.2%) of the strains had the *s1* signal. The *s1/m1* combination was observed in 22 (18.6%) strains, *s1/m2* was found in 16 (13.6%), and *s2/m2* was found in 72 (61.1%), while the remaining rare combination, *s2/m1*, was found in 4 (3.3%) strains. A single mosaicism combination of the *s/m* region was observed in 114 (96.6%) of the strains studied, while a colonization with a double *vacA* mosaicism combination was detected in 4 (3.3%) cases. The distribution of *vacA* genotypes according to *cagA* gene status is presented in Table 1. As expected, the *s2/m2 vacA* genotype was more frequently detected in *H. pylori cagA*-negative strains than in *cagA*-positive strains (93.2% and 6.8%, respectively; *P* < 0.0001).

The distribution of *H. pylori* genotypes in relation to clarithromycin resistance showed that *H. pylori* strains that were *cagA* positive were more often susceptible to clarithromycin (81.8% versus 53.4% for *cagA*-negative strains; *P* < 0.001), as shown in Table 2. Furthermore, as expected, clarithromycin resistance in *H. pylori* isolates was strongly associated with *vacA s2/m2* (38.9% and 13.1%, respectively; *P* < 0.001).

Relationship of clarithromycin resistance with host factors.

The results of analysis of *H. pylori* clarithromycin resistance in relation to age, place of birth, prior treatment, and gender of the patient from whom the *H. pylori* strain was isolated are shown in Table 3.

The results for *H. pylori* isolates with different clarithromycin susceptibility patterns according to patient age showed that *H. pylori* strains recovered from children were more often resistant to clarithromycin than strains isolated from adults (40.0% and 17.5%, respectively; *P* = 0.003).

We found a relationship between clarithromycin susceptibility in the *H. pylori* isolates and the place of birth of the patients from whom the bacteria were isolated. *H. pylori* strains isolated

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

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

^a For the values of 81.8% versus 53.4%, *P* < 0.001 (chi-square test).

from patients born in Spain were more often resistant to clarithromycin than strains isolated from patients born outside Spain (33.7% and 14.3%, respectively; *P* = 0.05).

The analysis of patients previously treated and those never treated for *H. pylori* infections confirmed that *H. pylori* isolates from previously treated patients were more often resistant to clarithromycin than *H. pylori* strains isolated from patients who had not received any treatment (58.3% and 21.5%, respectively; *P* < 0.0002).

Analysis of a possible association between *H. pylori* clarithromycin resistance and the gender of the patient showed that females and males were 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

TABLE 3. Association between clarithromycin susceptibility and *cagA* status, age, place of birth, and prior treatment

Characteristic	No. (%) of patients			
	Total (<i>n</i> = 117)	Clarithromycin susceptible (<i>n</i> = 75)	Clarithromycin resistant (<i>n</i> = 34)	Discrepant (<i>n</i> = 8)
Children	60	30 (50.0)	24 (40.0)	6 (10.0)
Adults	57	45 (78.9)	10 (17.5)	2 (3.5)
Spanish	89	53 (59.6)	30 (33.7)	6 (6.7)
Not Spanish	28	22 (78.6)	4 (14.3)	2 (7.1)
Prior treatment	24	8 (33.3)	14 (58.3)	2 (8.3)
No prior treatment	93	66 (71.0)	20 (21.5)	7 (7.5)

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 those 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.

ACKNOWLEDGMENTS

This study was supported in part by Fondo de Investigaciones Sanitarias grants FIS 05/2442 and FIS 05/2452 and an NIH grant (grant p60MD000538-05) to the NYU Center for the Study of Asian American Health.

REFERENCES

- Agudo, S., T. Alarcón, L. Cibrelus, P. Urruzuno, M. Martínez, and M. López-Brea. 2009. High percentage of clarithromycin and metronidazole resistance in *Helicobacter pylori* clinical isolates obtained from Spanish children. *Rev. Esp. Quimioter.* **22**:88–92.
- Ahmad, N., W. R. Zakaria, S. A. Abdullah, and R. Mohamed. 2009. Characterization of clarithromycin resistance in Malaysian isolates of *Helicobacter pylori*. *World J. Gastroenterol.* **15**:3161–3165.
- Alarcón, T., D. Domingo, M. J. Martínez, and M. López-Brea. 1999. *cagA* gene and *vacA* alleles in Spanish *Helicobacter pylori* clinical isolates from patients of different ages. *FEMS Immunol. Med. Microbiol.* **24**:215–219.
- Álvarez, A., J. Moncayo, J. Santacruz, M. Santacoloma, L. Corredor, and E. Reinos. 2009. Antimicrobial susceptibility and mutations involved in clarithromycin resistance in *Helicobacter pylori* isolates from patients in the western central region of Colombia. *Antimicrob. Agent Chemother.* **53**:4022–4024.
- Andersen, L. P., and T. Wadstrom. 2001. Basic bacteriology and culture, p. 947–961. In H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori*: physiology and genetics. ASM Press, Washington, DC.
- Atherton, J. C., T. L. Cover, R. J. Twells, M. R. Morales, C. J. Hawkey, and M. J. Blaser. 1999. Simple and accurate PCR-based system for typing *vacA* and *ureA* alleles of *Helicobacter pylori*. *J. Clin. Microbiol.* **37**:2979–2982.
- Boyanova, L., R. Markovska, D. Yordanov, M. Marina, K. Ivanova, S. Panayotov, G. Gergova, and I. Mitov. 2009. High prevalence of virulent *Helicobacter pylori* strains in symptomatic Bulgarian patients. *Diagn. Microbiol. Infect. Dis.* **6**:374–380.
- Cambau, E., V. Allerheiligen, C. Coulon, C. Corbel, C. Lascos, L. Deforges, C. J. Soussy, J. C. Delchier, and F. Megraud. 2009. Evaluation of a new test, genotype HelicoDR, for molecular detection of antibiotic resistance in *Helicobacter pylori*. *J. Clin. Microbiol.* **7**:3600–3607.
- Chey, W. D., and B. C. Wong. 2007. American College of Gastroenterology guideline on the management of *Helicobacter pylori* infection. *Am. J. Gastroenterol.* **102**:1808–1825.
- Clinical and Laboratory Standards Institute. 2009. Performance standards for antimicrobial susceptibility testing: 17th informational supplement. M100-S17. Clinical and Laboratory Standards Institute, Wayne, PA.
- De Francesco, V., M. Margiotta, A. Zullo, C. Hassan, F. Giorgi, M. Zotti, G. Stoppino, A. Bastianello, Diterlizzi, G. Verderosa, S. Morini, C. Panella, and E. Ierardi. 2009. *Helicobacter pylori vacA* arrangement and related diseases: a retrospective study over a period of 15 years. *Dig. Dis. Sci.* **54**:97–102.
- De Francesco, V., M. Margiotta, A. Zullo, C. Hassan, N. Valle, O. Burattini, D'Angelo, R. G. Stoppino, U. Cea, F. Giorgio, R. Monno, S. Morini, C. Panella, and E. Ierardi. 2006. Clarithromycin resistance and *Helicobacter pylori* genotypes in Italy. *J. Microbiol.* **44**:660–664.
- De Francesco, V., A. Zullo, E. Ierardi, and D. Vaira. 2009. Minimal inhibitory concentration (MIC) values and different point mutations in the 23S rRNA gene for clarithromycin resistance in *Helicobacter pylori*. *Dig. Liver Dis.* **41**:610–611.
- Elitsur, Y., Z. Lawrence, H. Rüßmann, and S. Koletzko. 2006. Primary clarithromycin resistance to *Helicobacter pylori* and therapy failure in children: the experience in West Virginia. *J. Pediatr. Gastroenterol. Nutr.* **42**:327–328.
- Evans, D. J., and D. G. Evans. 2001. *Helicobacter pylori* CagA: analysis of sequence diversity in relation to phosphorylation motifs and implications for the role of cagA as a virulence factors. *Helicobacter* **6**:187–198.
- Fariña, N., E. Kasamatsu, M. Samudio, M. Morán, R. Sanabria, and F. Laspina. 2007. Antimicrobial susceptibility of *H. pylori* strains obtained from Paraguayan patients. *Rev. Med. Chil.* **135**:1009–1014.
- Fox, J., and F. Megraud. 2007. *Helicobacter*, p. 947–961. In P. R. Murray, E. J. Baron, M. A. Pfaller, J. H. Tenover, and R. H. Tenover (ed.), *Manual of clinical microbiology*, 9th ed. ASM Press, Washington, DC.
- Glupczynski, Y. 1996. Culture of *Helicobacter pylori* from gastric biopsies and antimicrobial susceptibility testing, p. 17–32. In A. Lee and F. Megraud (ed.), *Helicobacter pylori*: techniques for clinical diagnosis and basic research. W. B. Saunders Company Ltd., Baltimore, MD.
- Horiki, N., F. Omata, M. Uemura, S. Suzuki, N. Ishii, Y. Iizuka, K. Fukuda, Y. Fujita, M. Katsurahara, T. Ito, G. E. Cesar, I. Imoto, and Y. Takei. 2009. Annual change of primary resistance to clarithromycin among *Helicobacter pylori* isolates from 1996 through 2008 in Japan. *Helicobacter* **14**:86–90.
- Kim, J. M., J. S. Kim, N. Kim, Y. J. Kim, I. Y. Kim, L. Y. Chee, C. H. Lee, and H. C. Jung. 2008. New mutations of 23S rRNA associated with clarithromycin resistance in *Helicobacter pylori* strains isolated from Korean patients. *J. Microbiol. Biotechnol.* **18**:1584–1589.
- Koletzko, S., F. Richy, P. Bontems, J. Crone, N. Kalach, M. L. Monteiro, F. Gottrand, D. Celinska-Cedro, E. Roma-Giannikou, G. Orderda, S. Kolacek, P. Urruzuno, M. J. Martínez-Gómez, T. Casswall, M. Ashorn, H. Bodanszky, and F. Megraud. 2006. Prospective multicentre study on antibiotic resistance of *Helicobacter pylori* strains obtained from children living in Europe. *Gut* **55**:1711–1716.
- Liu, Z., P. Zheng, and P. Yang. 2008. Efflux pump gene *hefA* of *Helicobacter pylori* plays an important role in multidrug resistance. *World J. Gastroenterol.* **14**:5217–5222.
- López-Brea, M., J. Martínez, D. Domingo, and T. Alarcón. 2001. A 9 year study of clarithromycin and metronidazole resistance in *Helicobacter pylori* from Spanish children. *J. Antimicrob. Chemother.* **48**:295–297.
- Malfertheiner, P., F. Megraud, C. O'Morain, F. Bazzoli, E. El-Omar, D. Graham, R. Hunt, T. Rokkas, N. Vakil, and E. Kuipers. 2007. Concepts in the management of *Helicobacter pylori* infection. The Maastricht III Consensus Report. *Gut* **56**:772–781.
- Mana, F. 2009. The Maastricht III consensus: summary and comments. *Acta Gastroenterol. Belg.* **72**:344–349.
- Mégraud, F., and P. Lehours. 2007. *Helicobacter pylori* detection and antimicrobial susceptibility testing. *Clin. Microbiol. Rev.* **20**:280–322.
- Mégraud, F. 2004. *H. pylori* antibiotic resistance: prevalence, importance and advantages in testing. *Gut* **53**:1374–1384.
- O'Connor, A., J. Gisbert, and C. O'Morain. 2009. Treatment of *Helicobacter pylori* infection. *Helicobacter* **14**:46–51.
- Panayotopoulou, E., D. Sgouras, K. Papadakis, A. Kalliaropoulos, G. Papathodoridis, A. Mentis, and A. Archimandritis. 2007. Strategy to characterize the number and type of repeating EPIYA phosphorylation motifs in the carboxyl terminus of CagA protein in *Helicobacter pylori* clinical isolates. *J. Clin. Microbiol.* **45**:488–495.
- Perez Aldana, L., M. Kato, S. Nakagawa, M. Kawarasaki, T. Nagasako, T. Mizushima, H. Oda, J. Kodaira, Y. Shimizu, Y. Komatsu, R. Zheng, H. Takeda, T. Sugiyama, and M. Asaka. 2002. The relationship between consumption of antimicrobial agents and the prevalence of primary *Helicobacter pylori* resistance. *Helicobacter* **7**:306–309.
- Rimbara, E., T. Kawai, and M. Sasatsu. 2008. Novel mutation in 23S rRNA that confers low-level resistance to clarithromycin in *Helicobacter pylori*. *Antimicrob. Agents Chemother.* **52**:3465–3466.
- Shanks, A. M., and E. M. El-Omar. 2000. *Helicobacter pylori* infection, host genetics and gastric cancer. *J. Dig. Dis.* **10**:157–164.
- Sugimoto, M., and Y. Yamaoka. 2009. Virulence factor genotypes of *Helicobacter pylori* affect cure rates of eradication therapy. *Arch. Immunol. Ther.* **57**:45–56.
- Taneike, I., A. Nami, A. O'Connor, N. Fitzgerald, P. Murphy, A. Qasim, H. O'Connor, and C. O'Morain. 2009. Analysis of drug resistance and virulence-factor genotype of Irish *Helicobacter pylori* strains: is there any relationship between resistance to metronidazole and cagA status? *Aliment. Pharmacol. Ther.* **30**:784–790.
- Tanuma, M., E. Rimbara, N. Noguchi, S. Boonyaritiikij, K. Kuwabara, Y. Fukunaga, and M. Sasatsu. 2009. Analysis of clarithromycin resistance and cagA status in *Helicobacter pylori* using feces from children in Thailand. *J. Clin. Microbiol.* **47**:4144–4145.
- Taylor, D. E., Z. Ge, D. Purych, T. Lo, and K. Hiratsuka. 1997. Cloning and sequence analysis of two copies of a 23S rRNA gene from *Helicobacter pylori* and association of clarithromycin resistance with 23S rRNA mutations. *Antimicrob. Agents Chemother.* **41**:2621–2628.
- Vallejos, C., L. Garrido, D. Cáceres, A. M. Madrid, C. Defilippi, C. Defilippi, and H. Toledo. 2007. Prevalence of metronidazole, clarithromycin and tetracycline resistance in *Helicobacter pylori* isolated from Chilean patients. *Rev. Med. Chil.* **135**:287–293.
- van Doorn, L. J., P. M. Scheneberger, N. Nouban, A. P. Plaisier, W. G. V. Quint, and W. A. de Boer. 2000. Importance of *Helicobacter pylori* cagA and vacA status for the efficacy of antibiotic treatment. *Gut* **46**:321–326.