Helicobacter pylori resistance to metronidazole, clarithromycin and amoxicillin in Colombian patients

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Summary

Helicobacter pylori (H. pylori), is a universal pathogen that infects more than half the world population. In the last two decades, the recommended treatment for its eradication, as first-line scheme is the standard triple therapy, consisting of an inhibitor of the proton pump, clarithromycin and amoxicillin or metronidazole. In recent years the effectiveness of this therapy has declined, especially due to the resistance of bacteria to metronidazole and clarithromycin.

Objectives: In this study, we evaluated the prevalence of primary resistance of Colombian *H. pylori* isolates to metronidazole, clarithromycin, amoxicillin. In addition, the *vacA* and *cagA* genotypes of strains isolated were determined and associated to correlate the virulence markers and antibiotic resistance. Methods: Minimum inhibitory concentration (MIC) for metronidazole, clarithromycin and amoxicillin were determined by E-test method. Genomic DNA was extracted, and allelic variants of *vacA* and *cagA* were identified by the polymerase chain reaction (PCR). Results: Resistance to metronidazole was 81.01 % (IC95% 70.3%-88.6%), to amoxicillin 3,8% (IC 95% 0-8,6%), and to clarithromycin 17.72% (IC95% 10.37-28.29). No significant correlation between pathogenicity and resistance or susceptibility was detected when MIC values for each antibiotic were compared with different *vacA* and *cagA* genotypes. Conclusion: We find a high rate of resistance to three principal antibiotics used in the majority of the successful schemes of eradication of the infection, which implies the need to investigate with priority new schemes of treatment for the eradication of the infection in Colombia.

Key words

Helicobacter pylori, genotypes, clarithromycin, amoxicillin, metronidazole.

INTRODUCTION

Helicobacter pylori (H .pylori) is a universal pathogen that infects more than half the population of the world (1, 2). It is the main etiological agent of chronic gastritis, peptic ulcers, gastric MALT lymphoma and gastric adenocarcinoma (1-3). Nevertheless, final consequences of the infection depend upon the host's genetic factors, external environmental factors and upon which H. pylori genotypes are present and how virulent they are, e.g. cag A (+) or vacA A s1, m1 (4, 5).

In the last two decades the recommended first-line treatment scheme for its eradication has been standard triple therapy. This consists of a proton pump inhibitor, amoxicillin and clarithromycin or metronidazole (6-8). Although initially the effectiveness of this traditional scheme was 90% (9-11), it has progressively diminished in many parts of the world. Its effectiveness is currently between 57% and 73% when treatment duration is seven days and 67% to 79% when treatment lasts 10 days (10). Effectiveness increases approximately 6% when treatment is extended to ten days from seven days, but this is still less than 80% and does not achieve optimal results.

It is believed that the decrease, which is now found consistently, is mainly due to increasing primary resistance of H. pylori to amoxicillin, clarithromycin and metronidazole (8-12). For this reason it is important to evaluate the prevalence of H. pylori's primary resistance to these three

key antimicrobials, which are the structure of the standard triple therapy that is still recommended as the first-line therapy of choice (8, 9). These antibiotics should be used with caution when local resistance to them is above 15-20% for clarithromycin and 40% for metronidazole at which values their effectiveness is considered to be compromised (9). Since one or more of these antibiotics is used in the majority of schemes that have been successful in the eradication of *H. pylori* (9, 10), it has become necessary to determine levels of resistance to these antibiotics and use that information in planning the choice of antimicrobials in clinical practice (11).

More than a decade ago, here in our environment, a group found an 82% resistance rate to metronidazole by using the E-test (13). It should be noted that the E-test can overestimate H. pylori's resistance rate to metronidazole compared to the resistance shown by the agar dilution technique which is considered to be the gold standard for determining this resistance (14). The prevalence of primary resistance to clarithromycin and amoxicillin remains unknown until the relationship between diverse H. pylori genotypes and resistance to antimicrobials is studied. As far as we could find, there had been no such study undertaken in our country, so we decided to do this study with the following objectives:

1. To determine the prevalence of H. pylori's primary resistance to three antibiotics that are considered to be the most important for eradication therapies: clarithromycin, metronidazole and amoxicillin.
2. To establish if the cagA and vacA H. pylori positive genotypes, and the different subtypes of the latter, are associated with resistance to different antimicrobials.

MATERIALS AND METHODS

A prospective and analytical study of prevalence was performed by the Gastroenterological Unit of the Fundadores Clinic and the Department of Microbiology in the Faculty of Sciences at the Pontificia Universidad Javeriana, both of which are in Bogotá, Colombia. This study was conducted between January 2008 and June 2009. The study prospectively included patients over the age of 18 years old that had been referred for an upper endoscopy at the Clínica Fundadores. The reasons for referral were dyspepsia or symptoms of gastro esophageal reflux that had not been previously treated for the eradication of H. pylori. Also, to be included in this study patients could not have received antibiotics or bismuth salts during the previous year, nor could they have taken antisecretory drugs for at least a month prior to performance of endoscopy. Before entering the study, but after receiving a complete and detailed explanation informed consent and the study, all patients signed an informed consent form. The research protocol and the informed consent form were both approved by the ethics and investigation committee of the institution where the study took place.

Exclusion criteria

Exclusion criteria included the following serious comorbidities: congestive heart failure (CHF), cerebrovascular accident (CVA), decompensated diabetes, coagulation abnormalities, cirrhosis, previous gastric surgery, pregnancy, lactation, drug or alcohol addiction, psychiatric illnesses, cancer and HIV infection. Other exclusion criteria included treatment with anticoagulants and chemotherapy.

All patients' endoscopies were performed in the morning after a minimum of six hours of fasting. Endoscopies were performed with patient in left lateral decubitus position, in the usual manner (15) following general hygiene recommendations for endoscopies (15). No sedation was used, but all patients received two applications (20 mg) of lidocaine spray (Lidocaine, topical solution, Ropsohn Therapeutics) to anesthetize the pharynx. The equipment used for EVDAs was an Olympus Exera CV 145. During the upper endoscopy, six biopsies were taken from the antrum: each two centimeters from the pylorus, three from the greater curvature and three from the lesser curvature. Six more biopsies were taken from the corpus: eight centimeters from the cardia, three from the lesser curvature and three from the greater curvature. Biopsies were preformed according to the protocol recommended by experts (16, 17).

From among these biopsy samples, two from the antrum and two from the corpus were used for histological study and determination of *H. pylori* infection through hematoxylin and eosin (HE) and Giemsa stain (when HE was negative). Two of the antrum and two of the body biopsies were used for H. pylori cultures. The other two biopsies from the antrum and the other two biopsies from the corpus were kept for use in new cultures in the event of unforeseen difficulties with the first cultures (contamination of the culture medium, poor quality of batch ingredients, etc.).

An additional biopsy of the antrum was taken for the rapid urease test, and further biopsies were taken if there were endoscopic lesions that warranted them (gastric ulcers, masses, elevations, tumors, etc.). We prepared the rapid urease test following recommended procedures (18). Demographic and other variables included in the study were entered prospectively and in the standard manner.

H. pylori culture and susceptibility testing of antibiotics in itro

Transport Procedure: Each biopsy taken during an upper endoscopy was placed in a cryovial with 500 µl of Brucella broth and was kept in cold chain until processed.

Procedure for Isolating *Helicobacter pylori*. Biopsies were macerated with a sterilized wooden applicator under total asepsis and sterility. The applicator had previously been treated in a 1% activated carbon solution. Biopsies were macerated until a homogeneous solution was obtained (19). Then, using a disposable inoculation loop, we planted the culture in Wilkins Chalgren medium modified for *H. pylori* and supplemented with Isovitalex and antibiotics. Once seeding had been done, the Petri dishes were placed into anaerobic jars. Then a microaerophilic atmosphere was generated with CampyPak packs (BBL – Becton, Dickinson and Co.). The cultures were incubated at a 37 degrees Celsius temperature for 4 – 15 days (19-20).

Identification tests for *Helicobacter pylori*: In order to verify the presence of *Helicobacter pylori* in the cultures, the following tests were performed (20): Gram stain: Small gram-negative curved bacilli; Catalase Test: Positive Catalase; Oxidase: Positive oxidase; Urease: Positive urease.

After biochemical test verification we proceeded to evaluate susceptibility to metronidazole, amoxicillin and clarithromycin. In addition, 60 of the 79 isolates were subjected to DNA extraction and amplification of the virulence genes vacA and cag A by PCR technique (19).

Genotypification of the cagA Gene by PCR (19, 21, 22)

To genotype the cagA gene we obtained products for DNA amplification by PCR in a final volume of 25 µl. For this we dispensed: 0.1 µl of Taq polymerase (TucanTaq - Corpogen), 2.5µl of buffer Taq (TucanTaq - Corpogen), 1.5 µl of $MgCl_2$ (TucanTaq - Corpogen), 0.5 µl of dNTPs mix (Invitrogen), 1 µl of each cagA Forward and Reverse primer (IDT – Coralville - USA), and 5 µl of DNA solution at a 100ng concentration. We completed this with molecular grade water for a final volume of 25 µl. The sequences of the cagA primers were:

cagA F(+) 5'- TTGACCAACAACCACAAACCGAAG - 3'
cagA R(-) 5'- CTTCCCTTAATTGCGAGATTCC – 3'

Positions are in accordance with the ORF *cagA* in Genbank sequence L11714.

The cagA amplification was performed in a thermocycler (MyCycler thermal cycler – BIORAD), as follows:

1. Initial denaturation: 9 minutes at 94°C.
2. 40 cycles of: Denaturation at 95°C for 30 seconds, hybridization at 50°C for 45 seconds and extension at 72°C for 45 seconds.
3. Final extension: 72°C for 5 minutes. After 5 minutes the amplicons were run through 2 % agarose gels and revealed in ethidium bromide solution.

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vacA amplification was performed in a thermocycler (MyCycler thermal cycler – BIORAD) as follows:

1. 35 cycles of: Denaturation at 94°C for 1 minute, hybridization at 52°C for 1 minute and extension at 72°C for 1 minute.
2. Final extension: at 72°C for 5 minutes.

H. Pylori genotypes were grouped into the "most virulent group" and the "less virulent group" (19). The first group was composed of cag A (+) and vacA (+) with subtypes s1am1 + . The second group was composed of cagA (-) vacA (+) but with s2m2 (+) subtypes. The sequence of the primers used is shown in the table 1.

Table 1. Sequence of the primers used.

Region	Primer	Sequence (5' 3')	Size and location
vacA m1	VA3-F VA3-R	GGTCAAAATGCGGTCATGG CCATTGGTACCTGTAGAAAC	290bp (2741-3030)
vacA m2	VA4-F VA4-R	GGAGCCCCAGGAAACATTG CATAACTAGCGCCTTGCAC	352bp (976-1327)
vacA s1	VA1-F VA1-R	ATGGAAATACAACAAACACAC CTGCTTGAATGCGCCAAAC	259bp (797-1055)
vacA s2	VA1-F VA1-R	ATGGAAATACAACAAACACAC CTGCTTGAATGCGCCAAAC	286bp (284-569)
vacA s1a	S1A-F VA1-R	GTCAGCATCACACCGCAAC CTGCTTGAATGCGCCAAAC	190bp (866-1055)
vacAs1b	SS3-F VA1-R	AGCGCCATACCGCAAGAG CTGCTTGAATGCGCCAAAC	187bp

DETERMINING THE MINIMUM INHIBITORY CONCENTRATION (MIC)

E-test Technique (23-25)

Starting with cultures which had been incubated for 2- to 3 days, suspensions were prepared in Brucella broth adjusted to 2 on the MacFarland scale (1 x 108 CFU / ml). The suspension was inoculated with a sterile swab on Mueller-Hinton agar plates and supplemented with 10% of horse serum and 2% of Isovitalex. We used a separate culture medium dish for each antibiotic (metronidazole, clarithromycin, amoxicillin) to be tested. Strips of E-Test ® (Biomeriux) were placed on culture medium plates inoculated with bacteria and then incubated at 37 ° C under microaerophilic conditions for 48-72 hours. Isolates were considered to be resistant if the minimum inhibitory concentration (MIC) was found in levels at or above 8 µg/ml for metronidazole, 0.5 µg/ml for clarithromycin, and 1µg/ml for amoxicillin (23). We used H. pylori control strain NCTC 11637 to control culture mediums and E-test strips (24, 25).

STATISTICAL ANALYSIS

With the data obtained we developed a database using EPI INFO 6.0. The results obtained were statistically analyzed and processed with STATA 6.0. For each isolate we analyzed the percentage of resistance to clarithromycin, amoxicillin and metronidazole and determined the percentage presence of cagA gene and different alleles for *vacA gene*. We searched for associations between the presence of virulence genes and antibiotic resistance which were was evaluated by chi-square tests (X^2) with an alpha value (α) of 0.05.

RESULTS

We were able to obtain 79 H. pylori isolates out of 99 samples from 99 patients. The H. pylori diagnosis for these patients was documented with the rapid urease test and positive identification of H. pylori through histology. The recovery rate was with 80%. 67 % of the patients in which the microorganism was recovered were women. Average age of the total sample was 54 +/-15 years old. 16 patients (25%) were endoscopically diagnosed with erosive esophagitis, 79 patients (80%) were endoscopically diagnosed with chronic corporal and antral gastritis, and for 4 patients (5%) were endoscopically diagnosed with duodenal ulcers. According to E-Tests, prevalence of resistance was 81.01% for metronidazole (IC 95%70.31-88.64), 17.72% for clarithromycin (IC95% 10.37-28.29%), and 12.75% for amoxicillin (IC95% 6.56-22.5%) (See Table 2).

Table 2. Prevalence of resistance to different antibiotics.

Clarithromycin	Amoxicilin	Metronidazole
E-TEST	E-TEST	E-TEST
14/79	3/79	64/79
17,72% IC 95% 10,37-28,29%	3,8% IC 95% 0-8,6%	81,01% IC 95% 70,31-88,64

There were no statistically significant differences in rates for the three antibiotic resistances among men and women. Table 4 shows the characterization of the genotypes in 60 isolates and the prevalence of resistances to the antimicrobials studied.

25% of the genotypes identified were part of the "more virulent" group, as shown in table 4. The table also shows relative frequencies of the rest of H. pylori genotypes in the 60 patients examined. No statistically significant differences were found between cagA vacAs1am1 and other genotypes in terms of resistances to clarithromycin, amoxicillin and metronidazole. (P=0.36, p=0.36 and p=1 respectively, Table 3 and Table 4.)

DISCUSSION

For gastroenterologists and primary care physicians, the eradication of H. pylori is currently a great challenge because of the microorganism's increasing primary resistance to the antibiotics most frequently used for its treatment (8-12,27). Antibiotic resistance is partly due to a population's exposure to them as monotherapies for various infectious diseases (11 and 12). The recent Third Maastricht Consensus (9) recommended continued use of triple therapy for seven days in populations with less that 15-20% resistance to Clarithromycin. When resistance is more than 20%, treatment should be prolonged to 14 days or a quadruple therapy including bismuth should be used for 10 to 14 days. Use metronidazole was also recommended in triple therapy when resistance is less than 40%.

In this study we found that primary resistance to metronidazole was 81.01% and that primary resistance to clarithromycin was 17.72%. Both are above the suggested cut off limits above which their use in the triple therapy as first-line scheme should be avoided (9).

Our findings regarding resistance to metronidazole are consistent with findings in other developing countries (28, 29), and with other Colombian studies in which the same E-test methodology was used (13, 30). In 1998, Gutiérrez et al (13) found 82% resistance to metronidazole. Recently,

Table 3. Proportion of Resistance to Clarithromycin, Amoxicillin and Metronidazole in H. Pylori Genotypes.

Genotype	Clarithromycin resistance		Amoxicilin resistance		Metronidazole resistance	
	N	% IC95%	n	% IC95%	n	% IC95%
cagA(+) vacAs1m1	5/17	29,4% (7-51)	1/17	5,8% (0-17)	12/17	70,5% (48-92)
cagA(+)vacA s2m2	0/4	-	1/4	25% (0-79)	1/4	25% (0-79)
cagA(+) other vacA subtypes	2/11	18% (0-45)	3/11	27% (0-58)	8/11	72% (46-99)
cag A (-)vacAs2m2	2/11	18% (0-45)	1/11	9% (0-26)	8/11	72% (46-99)
cagA(-) vacAs1/m1	0/5	-	1/5	20% (0-65)	5/5	100% (90-100)
cagA(-) other vacA subtypes	4/12	33% (2-64)	2/12	16% (0-41%)	9/12	75% (46-100)

Table 4. Evaluation of resistance relation to clarithromycin, amoxicillin and metronidazole against different genotypes of virulence H. pylori.

	Clarithromycin	Amoxicilin	Metronidazole
cagA(+) vacAs1m1 vs other cag(+) strains	NS (p=0,27)	NS (p=0,35)	NS (p=0,52)
cagA(-) vacAs2m2 vs other cag(-) strains	NS (p=0,64)	NS (p=0,52)	NS (p=0,54)
cagA(+) strains vs cag(-) strains	NS (p=0,96)	NS (p=0,88)	NS (p=0,26)

*Statistical significance was evaluated with 0.05 alpha.

Henao et al found 72% resistance (30). The high level of resistance found in this study contrasts with 33% in European countries (31), 39% in the United States (32), 32% in Australia and 4% in Japan (33). The E-test technique may overstate resistance to metronidazole, as rates shown in the agar dilution technique (14) can be 10-20% less (12). Thus, we still believe that at such high rates of resistance to metronidazole the predictive value of the E-test results will be above 40%. Experts consider this percentage the maximum limit for the use of the medication (9).

Prevalence of resistance to metronidazole was similar among men and women (79.5% and 82.7% respectively).

The 17.7% resistance to clarithromycin is similar to the 15% resistance that Henao and colleagues published this year (34), and contrasts sharply with the 3.8% found by other researchers in mid-western Colombia (35). It is possible that the discrepancy in the results of the last study is related to the socio economic status of the population studied. As the authors express, these people probably have less exposure to antimicrobials since they are not in the mandatory Colombian health plan. However, up until now it has been considered that resistance to antimicrobials is fundamentally related with previous use of macrolides for the treatment of respiratory infections (12). Similarly, our findings differ from those in northern Europe and Scandinavia, where prevalence is 4% (12) and 1-3% (36) respectively and is higher than the 12.9% found in the United States (37). The overall prevalence of resistance to clarithromycin in Europe is 10%, while in southeastern Europe it is 18% (12). This is consistent with our results.

The impact of resistance to metronidazole and clarithromycin is transcendental in infection by H. pylori. Resistance to metronidazole reduces efficacy 50% in triple and quadruple therapies (27). Resistance to clarithromycin reduces it from 37% (38) to 70% (12). In France it has been found that when the strain is sensitive to clarithromycin, the eradication rate is 87.8%, but drops to 18.3% when there is resistance to it (12).

Maastricht still recommends (9) a first-line triple therapy with clarithromycin-metronidazole for 14 days or a quadruple therapy when there is more than 15% isolated resistance to clarithromycin and less than 40% isolated resistance to metronidazole.

However, there are no recommendations for geographical areas where there are simultaneously high resistance rates for both antibiotics, as we found in this study. The implication is that here in our environment there is an urgent need to investigate well tolerated therapies that overcome resistance to the two medications. A strategy might be the classic sequential 10 day therapy of a proton pump inhibitor with amoxicillin the first five days, and clarithromycin plus tinidazole for the last five days instead of amoxicillin. This does not substantially decrease its efficacy when there is resistance to clarithromycin but loses it when there is dual resistance to clarithromycin and metronidazole as was recently demonstrated in one of the most important studies

published (39). In this study no simultaneous resistance in the same strain was found; however, high resistance rates to metronidazole and clarithromycin raise doubts over the usefulness of the sequential therapy and should encourage priority research in our environment.

Based on our findings, another alternative would be to use triple therapies containing levofloxacin, which have shown efficacy in first-line therapies (40-42) as well as in second (43-46) and third-line rescue therapies (47). Resistance to amoxicillin has been found to be less than 2% worldwide and as a consequence, up until now is not considered a problem for the eradication of H. pylori (10 and 12). The 12.7% resistance found in this study is a shocking discovery that involves additional difficulties in the management of H. pylori in our environment. So far the countries with the highest resistance rate to the antimicrobial were Kenya with 4.6% resistance (48) and Bangladesh with 6.6% resistance (49).

We found no association between H. pylori genotypes and resistance to the three antibiotics in our study, which coincides with similar research done in other parts of the world (50-53). However, this differs from findings by Irish researchers who discovered that resistance rate to metronidazole is higher in cagA(+)vacAs1m1 strains (54).

Taking into account the results in this study, in which we found high resistance rates for the three most important antibiotics used eradicate *H. pylori,* the usefulness of the standard triple therapy as first-line scheme in our environment might be in question. However, the only way to confirm this assumption would be to conduct a clinical trial. Although further studies, preferably multi-centric, are required to confirm and extend the results of this study, we believe that the information derived from this study can be useful to physicians involved in the treatment of *H. pylori* for planning the choice of antibiotic scheme for use in empirical therapy. Experts believe that, as for treatment other infectious diseases, it is essential to have information on levels of resistance of the microorganism to commonly used antibiotics in order to use effective schemes for the treatment of H. pylori (10-12). In this respect this study has uncovered some troubling data.

In conclusion, we have found a high resistance rate to three main antibiotics used in most successful schemes for the eradication of the infection. This information has great impact in our country and thus might imply that preferential research for different therapies of H. pylori is needed.

Conflict of interest

This research was founded by Colciencias, as part of the project "Eradication of Helicobacter Pylori: Triple Therapy with Levofloxacin" Code 1203-408-20464.

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