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Antimicrobial susceptibility of *Helicobacter pylori* and mechanisms of clarithromycin resistance in strains isolated from patients in Uruguay

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ABSTRACT

The prevalence and mechanisms of antibiotic resistance of *Helicobacter pylori* have not yet been investigated in Uruguay. The objective of this study was to assess the susceptibility of *H. pylori* to the most frequently used antibiotics and to determine the mechanism of resistance to clarithromycin. Seventy-nine isolates were obtained from gastric biopsies of 50 adult patients during two periods, 2001 and 2006. The former group enrolled the general population (GP), the latter group Afro-descendant (AD) subjects. The minimum inhibitory concentrations of clarithromycin, amoxicillin, tetracycline, metronidazole, and levofloxacin were determined using the E-test technique. Amplification was achieved through PCR and nucleic acid sequencing to detect mutations in the site of action of clarithromycin in the rRNA gene 23S. No amoxicillin or tetracycline-resistant strains were found. Clarithromycin resistance was found in 12% of the patients overall: 19.4% resistance in AD patients and no resistance in the GP group. This difference was statistically significant. The highest resistance was seen with metronidazole (36%), present in similar proportions in the two groups: 36.8% (GP) and 35.5% (AD). One GP patient and one AD patient had levofloxacin-resistant strains. Sequencing analysis of gene 23S rRNA showed that only mutation in position 2143 was presented in all clarithromycin-resistant strains.

Key words: *H. pylori.* Clarithromycin resistance. Uruguay.

INTRODUCTION

Helicobacter pylori infection of the gastric mucosa is associated with chronic gastritis, gastro-duodenal ulcer, gastric adenocarcinoma and MALT lymphoma (1-3). Therefore, eradication of the infection is imperative as soon as the presence of the bacteria is confirmed in the setting of one of the above conditions in order to allow ulcers to heal and reduce the risk of developing gastric cancer (4,5).

Therapy is based on combinations of antimicrobials and proton pump inhibitors and is usually prescribed empirically, without any susceptibility testing. Although *H. pylori* may be susceptible to many antibiotics *in vitro,* in reality, very few agents can be used to treat the infection *in vivo*. Several therapy schemes have been evaluated for the eradication of *H. pylori*. Most consensus groups accept that so-called triple therapy (6) is the best choice available. Triple therapy consists of a proton-pump inhibitor combined with two antimicrobials, amoxicillin and clarithromycin as first line agents. However, using this regimen, therapy failures have been reported in up to 15-40% of cases (7-9). Other therapies available for patients with persistent infection include substituting the antimicrobials with metronidazole, tetracycline, or levofloxacin, or adding a bismuth-derived product (10). Ten-day sequential therapy: five days of treatment with a proton-pump inhibitor and amoxicillin followed by five days of treatment with the proton-pump inhibitor and two other antibiotics (usually clarithromycin and a 5-nitroimidazole) has also been proposed. A recently published meta-analysis shows it yields better results than standard triple therapy (11). This represents an interesting option in the effort to overcome the problem of resistance.

Although antimicrobial resistance is the leading factor responsible for eradication failure, there are other contributing causes, such as a patient's lack of compliance,

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inadequate length of therapy, or a high bacterial burden. Also, the therapeutic effects of omeprazole are assumed to depend on CYP2C12 genotype status of the host (12), although this has not been confirmed in a group of Latin American patients (13)

H. pylori's antimicrobial resistance rates vary by drug due to mutations that cause changes in each drug's site of action. The special nutritional and atmospheric conditions required by these organisms make susceptibility testing methods relatively difficult; however, the E Test technique developed for determining the minimum inhibitory concentration has remained valid (14). There is a broad range of resistance variability depending on the drug. In the case of metronidazole, current resistance is very high, with values ranging from 40-90%, depending on geographic region (15,16). However, the clinical consequences of such resistance are not definitive since there is no evidence of a remarkable increase in the rate of therapy failure (15). With respect to clarithromycin, the resistance rates reported in Europe range from 1.7% in the Netherlands to 23.4% in Italy (16). They are higher in Latin America, reaching 24% in Mexico (17). High percentages (27%) of resistance to this drug are also reported in Japan (18). In this case, the clinical implications are significant, since a clear reduction in the response rates obtained with the classical plan has been demonstrated (19,20). With regard to fluoroquinolones, there are few resistance studies. Papers report rates ranging from 4-5% in France and the Netherlands and reaching up to 20% in Portugal (16). Low resistance is reported for amoxicillin and tetracycline, ranging from 0 to 1% (16). As there is no evidence of increasing resistance to amoxicillin, this drug continues to be used as first line therapy.

Because of its relevance as a public health and medical issue, it is important to know the local patterns of antibiotic susceptibility in order to enable prescribers to select the most adequate therapy. Although triple therapy is the most common clinical practice in Uruguay, there are no publications on antibiotic resistance of *H. pylori* isolated from Uruguayan patients.

The objectives of this study were to assess the susceptibility of the antibiotics most frequently used for the eradication of *H. pylori* (amoxicillin, clarithromycin, tetracycline, levofloxacin and metronidazole) and to determine the mechanism of resistance to clarithromycin occurring in strains isolated from patients in Uruguay.

MATERIAL AND METHODS

Study design and sample collection

The study included 79 strains isolated from 50 patients that sought care at the Department of Gastroenterology of the Clínicas Hospital in Montevideo. The exclusion criteria were a history of gastrectomy, active gastrointestinal bleeding, evidence of malignancy (except when limited to skin or prostate), and the use of antibiotics or proton pump inhibitors within 15 days of inclusion in the study. Strains were only collected from patients over 18 years of age in whom gastroscopy was indicated because of upper GI symptoms. All subjects signed a written informed consent agreeing to participate in the research. The protocol and the informed consent were evaluated and approved by the Clínicas Hospital's Independent Ethics Committee.

The biopsies were obtained during two periods. In the first period, 2001, the specimens were collected from the general population (GP). Results from that research have been already published (21). In the second period, 2006, the specimens were collected from a population of Afrodescendants (AD).

Prior to endoscopy, detailed data were obtained regarding each patient's clinical history, including age, gender, place of birth, recent use of antibiotics or acid secretion inhibitors, family and personal history of gastric cancer or gastro-duodenal ulcers, length and severity of the GI symptoms, as well as the reason for requesting endoscopic assessment.

All patients underwent esophagogastroduodenoscopy, including collection of biopsies of the gastric mucosa. Five specimens were taken from each patient: two from the body, one for histology and the other for culture, and three from the antrum, for rapid urease test (*Campylobacter*-like organism test), histology and culture.

The specimens to be cultured were stored at -70 ºC and subsequently sent to the Research Laboratory of the New York University's School of Medicine for processing. The biopsies were shipped in full compliance with the regulations in force for transportation of biological material.

Culture preparation

The biopsies were soaked and sowed in selective (Agar Skirrow) and non-selective (Tripticase Soy Agar with 5% sheep blood) culture media. They were incubated at 37 ºC for 7 days in microaerobiotic conditions. Gram straining, catalase, oxidase and urease tests were conducted in suspicious colonies. The colonies identified as *H. pylori* were frozen at -70 ºC in *Brucella* broth with 15% glycerol until the time of the study. The strains were thawed and isolated in a non-selective medium, and were re-sowed twice before the susceptibility studies.

Susceptibility testing

The minimum inhibitory concentration was determined using the E test technique (AB Biodisk, Sweden) as recommended by the manufacturer. The drugs tested were: amoxicillin (AMX), clarithromycin (CLA), tetracycline (TET), levofloxacin (LVX) and metronidazole (MET).

The inoculum was prepared in Muller Hinton broth, adjusting it to a turbidity equivalent to one in Mac Farland's scale; it was sowed with a swab in Tripticase Soy Agar with sheep blood and incubated at 37 ºC in a microaerobiotic atmosphere, except for metronidazole, which was incubated in anaerobiotic conditions the first 24 hours and then in microaerobiosis.

Plates were read the third day of incubation to define a strain as susceptible or resistant. The minimum inhibitory concentration was established in accordance with the manufacturer's recommendations. The clarithromycin-resistant strain of *H. pylori* (98-1007) isolated in Nashville was added as a control, to check the technique's efficacy.

The breakpoints used to classify strains as susceptible or resistant differed for each agent: AMX: susceptible ≤ 0.25 μg/mL; CLA ≤ 0.25 μg/mL; intermediate = 0.5 μg/mL; resistant ≥ 1 μg/mL; TET resistant > 2 μg/mL; LVX resistant > 1.0 μg/mL, and MET resistant > 8 μg/mL (22,23).

Clarithromycin resistance

A portion of 23S (rRNA gene) was amplified using PCR in order to study the mechanism of resistance to clarithromycin in all the resistant strains and a sample of the susceptible strains. The bacterial DNA of the strains to be tested was extracted using Wizard (Promega, Madison WI) – a DNA purification kit – and was stored at – 20 °C until processing. A pair of previously described primers (24) was used to amplify a fragment of 1143 base pairs. The amplified fragment was sequenced, and the base pairs sequence thus obtained was compared to the sequences of the same fragment in the gene of non-resistant strains.

Data analysis

The Fisher's exact test was used to compare variables.

RESULTS

Fifty out of the 79 strains isolated from antral and body biopsies came from 31 AD patients, and 29 strains came from 19 GP patients. In the AD group, 19 patients had two strains isolated from their gastric mucosa, one from the antrum and the other from the body, while 12 were colonized by only one strain, six from the body and six from the antrum. In the GP group, *H. pylori* was isolated from both antral and body biopsies in 10 patients, while nine patients showed only one strain, four antral and five only in the body.

No AMX-resistant strains were identified in either of the two groups. All the strains were classified as susceptible, since they showed minimum inhibitory concentrations (MIC) equal to or lower than 0.064 μg/mL. Ninety-five per cent of the strains showed an AMX MIC lower than 0.016 μg/mL.

In the case of CLA, seven resistant strains were found in six AD patients. This represented 14% of the AD strains isolated and 19.4% of the AD patients studied, respectively. No resistant strains were found in the GP group. This difference was statistically significant ($p = 0.03$).

No resistant strains were found in either of the two groups for TET. The highest MIC detected was 1 μg/mL.

The MIC values for LVX ranged from lower than 0.016 to 32 μg/mL. Three resistant strains were found. One was from an antral isolate of an AD patient with a MIC of 32 μg/mL, which was not consistent with the body isolate (which was susceptible), and two strains belonged to the same patient in the GP group.

MET showed the greatest resistance with this technique, present in 28 strains (11 strains in the GP group and 17 in the AD group). This represented 36.8% of the GP patients and 35.5% of the AD patients. There was also a lack of consistency between the sensitivity to this antibiotic in antral and body strains in six patients (five in the AD group and one in the GP group).

Resistance to more than one drug was observed in two patients in the AD group. In both cases, the simultaneous resistance involved CLA and MET. No resistance associations were observed in the GP group.

The Sequencing analysis of the 23S rRNA confirmed that the 7 clarithromycin resistant strains had the A2143G mutation, but none of the susceptible strains had the mutation (Fig. 1).

DISCUSSION

Failures in treatment of *H. pylori* infection may be due to several causes, including the key issue of resistance. *H. pylori* develops resistance by acquiring chromosomal mutations at the site where the drug acts (16).

It is interesting to highlight that susceptibility testing of multiple strains of one patient, obtained from various sites in the stomach mucosa, sometimes yield contradictory results. This fact has been pointed out by other authors, and is known as "inter-niche heteroresistance" (25,26); this supports the notion that the human being may be colonized with more than one strain of *H. pylori* (27).

In general, the *H. pylori* isolated from Uruguayan patients was consistent with strains studied in other developing countries worldwide. No AMX-resistant strains were observed in this study, a finding in line with most international papers, which show that the resistance of *H. pylori* to this drug is either very rare or non-existent (9).

The high MET-resistance rates shown in the Uruguayan population are similar to those found in other developing countries. This fact has been attributed to

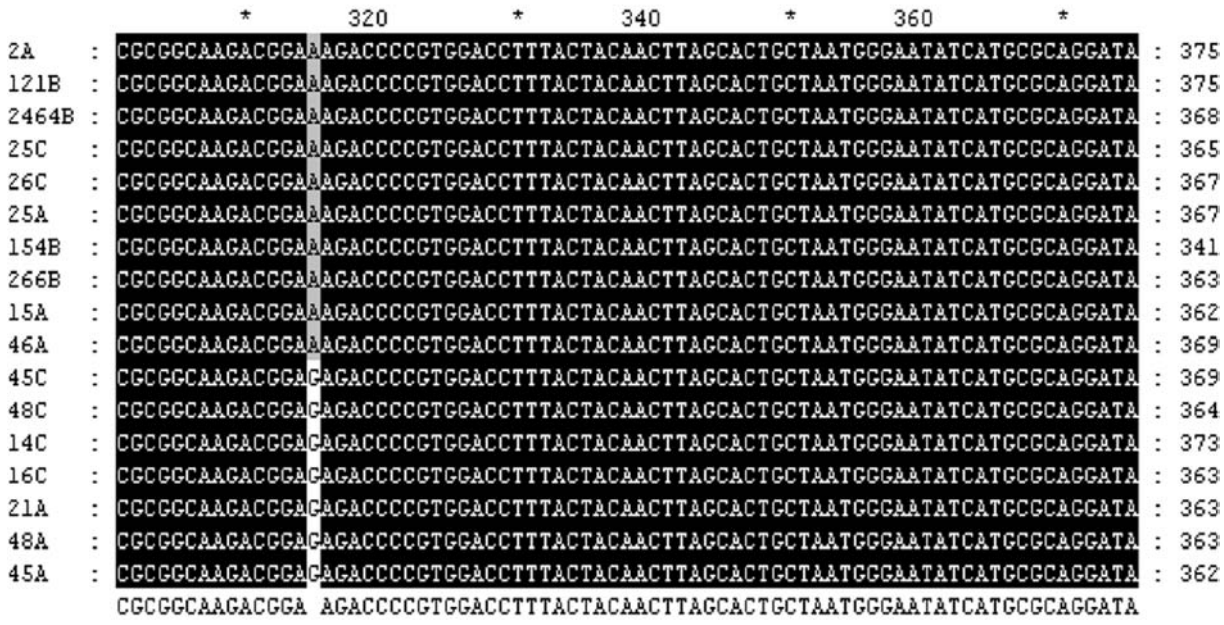

Fig. 1. Sequence of rRNA fragments. The first 10 strains are CLA sensitive, negative controls. The last 7 strains are CLA resistant. The position of the mutation is identical in all resistant strains, as shown.

frequent use of the drug, which is commonly prescribed for other diseases, especially parasitic conditions or gynecological infections (19,27). For this reason, MET has been excluded from first line empirical therapy plans. The results of *in vitro* resistance to this drug are also poorly correlated with the outcomes of therapy and consequently susceptibility testing is not routinely indicated (29).

No CLA resistant strains were found in the GP group but 14% of the strains isolated in the AD patients were resistant, corresponding to 19.4% of the patients. In spite of the relatively small number of strains included in this study, this difference was statistically significant ($p = 0.03$). Of note, this difference between strains was not significant when analyzed in terms of patients. The greater resistance in the AD strains may suggest an increased resistance to clarithromycin over the last five years, which could be due to the population's greater exposure to macrolides during the years elapsed between the data collections of the two groups. On the other hand, the ethnic differences between the populations enrolled in 2001 and 2006 could account for the discrepancy in resistance, with the Uruguayans of African descent having a greater level of resistance for environmental or genetic reasons. Further research would be necessary to distinguish between these two possibilities.

Importantly, the fact that all the strains tested were tetracycline susceptible and overall displayed a low resistance to levofloxacin suggests that these antibiotics would be valuable alternatives in the management of *H. pylori* infection in Uruguay.

With regard to the mechanisms of resistance studied in CLA using genotypic techniques, this study has documented the presence of mutations in 23S rRNA at position 2143, which has been described as the mechanism most frequently reported globally (30). Other mutations at position 2142 and position 2182 have been reported and are associated with higher levels of resistance (31). There was no evidence of these mutations in the *H. pylori* strains isolated from the Uruguayan patients studied.

Increased resistance to CLA over time has led to recommendations to test susceptibility before starting therapy in geographical areas with greater than 15-20% resistance to CLA (24). This study showed a resistance rate in the AD subset that fell into this category. Susceptibility testing could be implemented through culture and susceptibility tests; however, genotypic techniques permit detection of the organism as well as its virulence mechanisms and changes in virulence against this drug and others (32-34). For example, dual priming oligonucleotide-based multiplex PCR has been proposed to the detection of clarithromycin resistant strains of *H. pylori* in gastric samples (35). These techniques appear attractive due to rapid testing and the resultant low incidence of CLA resistance but they are costly and not readily available in Uruguay.

This study is the first report on antimicrobial susceptibility of strains of *H. pylori* isolated from Uruguayan patients, and it provides evidence of the occurrence of alterations in the site of action of clarithromycin in resistant strains. The rate of primary resistance to the drugs included in the guidelines for the empirical therapy is not high;

Table I. Antimicrobial resistance per isolate

	GP strains n = 29			*AD strains n = 50*			*Total*		
	S	*R*	*% resistance*	*S*	*R*	*% resistance*	*S*	*R*	*% resistance*
AMX	29	0	0	50	0	0	79	0	0
CLA	29	0	0*	43	7	14*	72	7	8.9
TET	29	0	0	50	0	0	79	0	0
LVX	27	2	6.9	49	1	2	76	3	3.8
MET	18	11	37.9	33	17	34	51	28	35.4

*p value = 0.03, Fisher's exact test.

Table II. Percentage of resistance per patient

	Strains in GP patients n =19			*Strains in AD patients n = 31*			*Total*		
	S	*R*	*% resistance*	*S*	*R*	*% resistance*	*S*	*R*	*% resistance*
AMX	19	0	0	31	0	0	50	0	0
CLA	19	0	0	25	6	19.4	44	6	12
TET	19	0	0	31	0	0	50	0	0
LVX	18	1	5.2	30	1	3.2	48	2	4
MET	12	7	36.8	20	11	35.5	32	18	36

however, and particularly for the case of metronidazole and clarithromycin, there is an increased resistance that is especially worrisome for clarithromycin, since it is one of the drugs used in the country's first line plan. In particular, it is important to determine whether the increased resistance to clarithromycin is a result of its increased use or due to the ethnical differences of the populations herein described. The genotypic susceptibility testing techniques are quite promising in terms of further research in that regard.

Continued surveillance of the resistance profiles and the mechanisms involved in resistance present in the strains of *H. pylori* isolated in Uruguay are essential if therapeutic plans are to fit the country's needs.

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