BRIEF COMMUNICATION

The interplay between mutations in *cagA*, *23S* rRNA, *gyrA* and drug resistance in *Helicobacter pylori*

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ABSTRACT

In this study, we evaluated the mutations of *Helicobacter pylori* associated with resistance to clarithromycin and levofloxacin. Furthermore, based on the proposed interaction between antimicrobial resistance and pathogenicity, we correlated the mutation profiles of the strains with the presence of the pathogenicity gene *cagA*. We analyzed 80 gastric biopsy specimens from *H. pylori*-infected patients for point mutations in the *23S* rRNA gene region and in the *gyrA* gene, which are related to clarithromycin and levofloxacin resistance, respectively, and investigated the presence of the *cagA* gene in these strains. We observed that in the assayed biopsies, 8.7% (7/80) had mutations in the *23S* rRNA gene region at positions 2143 and 2142, while 22.5% (18/80) had mutations in *gyrA* at codons 87 and 91. Moreover, absence of the CagA-EPIYA pathogenicity factor was observed in 68% (17/25) of resistant samples. The knowledge of the local profile of antimicrobial resistance and the complex interplay involving resistance and pathogenicity can contribute to an appropriate clinical approach.

KEYWORDS: Antimicrobial resistance. Point mutations. Clarithromycin. Levofloxacin. CagA EPIYA.

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INTRODUCTION

The successful treatment of patients infected with *Helicobacter pylori* can aid in prevention of serious gastroduodenal diseases, such as gastritis, peptic ulcers, adenocarcinoma and MALT (Mucosa-Associated Lymphoid Tissue). According to the Maastricht V Consensus, therapeutic regimens for eradication of this microorganism involve the use of combination therapy. Triple therapy, recommended as a first-line empirical treatment, uses a combination of amoxicillin (AMX) and clarithromycin (CLA) plus a proton pump inhibitor. However, in areas of high clarithromycin resistance (more than 15%), quadruple therapy with bismuth is the recommended first-line treatment. As a rescue option, the second-line treatment is based on the use of levofloxacin (LVX)[1].

A high failure rate of anti-*H. pylori* therapy is observed, primarily due to the acquisition of point mutations by *H. pylori* that results in resistance to the antimicrobials CLA and LVX. This mechanism of resistance is related to the frequent and/or inadequate use of antimicrobials, which can place selective pressure on resistant strains. Regarding CLA resistance, an association of CLA resistance with the presence of mutations in the *23S* rRNA region has been described, especially at position 2143 (A→G) and 2142 (A→G)[2]. The resistance of *H. pylori* to LVX is associated with the presence of mutations in the *gyrA* gene, especially at codon

Asn87 (substitution of asparagine by lysine) and Asp91 (substitution of aspartic acid by asparagine)[3].

In addition to the resistance mechanisms developed by *H. pylori* against these antimicrobials, some studies have noted that the presence or absence of certain pathogenicity genes of bacteria and the gender and age of the patients are risk factors in the development of antimicrobial resistance[4,5]. There is evidence that the presence of the *cag*A (*cytotoxin-associated gene* A) pathogenicity gene plays an important role in the induction of these severe gastric disorders. This is primarily due to the variable number of repetitive sequences of amino acids Glu-Pro-Ile-Tyr-Ala (EPIYA) in its 3' region, where it undergoes tyrosine phosphorylation, which may result in modulation of the inflammatory response[6].

Therefore, some studies have proposed the implementation of a molecular approach for gastric biopsy specimens of *H. pylori* in clinical practice since *H. pylori* exhibits slow growth and requires specific culture medium, making the phenotypic approach challenging and slow. Furthermore, molecular methods are capable of detecting the molecular basis of resistance and identifying pathogenicity genes[7,8].

Thus, in this study, we analyzed mutations in *23S* rRNA and *gyrA* genes, which are related to resistance of a component of the first choice therapy, clarithromycin (CLA) and to the antimicrobial used as an alternative to standard therapy, levofloxacin (LVX), respectively[2,3]. In addition, we correlated this resistance with the presence of the pathogenicity gene *cagA* and with epidemiological factors of the patients included in the study.

MATERIAL AND METHODS

We analyzed 80 gastric biopsy specimens previously identified as *H. pylori* positive, obtained from upper digestive tract endoscopies of patients at the Sao Francisco de Paula University Hospital, in the city of Pelotas, Brazil. All patients provided informed consent and responded to a questionnaire. This study was approved by the Ethics Committee of the Universidade Catolica de Pelotas (registration Nº 658.179).

H. pylori infection was determined by histological analysis or by an *in-house* urease test and was confirmed by polymerase chain reaction (PCR). The histological analysis of the gastric biopsy was performed by Hematoxylin-Eosin and Giemsa staining. For the *in-house* urease test, the biopsies were incubated in 1.0 mL of urea broth (Isofar, Brazil), prepared according to the manufacturer's instructions. The DNA was extracted from the biopsies, and the *glmM* gene was PCR amplified to confirm the *H. pylori* infection, as previously described by Vianna *et al.*[9].

The PCR-RFLP (Restriction Fragment Length Polymorphism) approach was used to investigate the mutations related to CLR resistance in the *23S* rRNA gene at positions 1342 to 1360 and 2765 to 2745 (complementary to the *Escherichia coli* homolog), which amplified an approximately 1,400 bp fragment. The fragment was digested with the restriction enzymes *Mbo*II and *Bsa*I at 37 °C for 2 h and 30 min, to detect mutations at A2142G and A2143G, respectively, as previously described[2]. The mutations related to LVX resistance were explored by assessing the sequences at *H. pylori* positions 752601 to 752620 and 753182 to 753163, which amplified an approximately 582 bp fragment of *gyrA*, as previously described[3]. The sequencing equipment was an ABI 3500 Genetic Analyzer, Life Technologies - Applied Biosystems, used according to the manufacturer's instructions. Sequence alignment was performed using the BioEdit Sequence Alignment Editor, and sequences were compared to the genome of *H. pylori* 26695 by BLAST analysis.

The presence of the *cagA* gene was assessed by amplification of a variable region in the 3' portion of the coding region. The reactions yielded products of 500-850 bp as follows: EPIYA AB, 500 bp; EPIYA-ABC, 640 bp; EPIYA-ABCC, 740 bp; and EPIYA-ABCCC, 850 bp, as previously described[6,9].

Statistical analysis was performed by using the Fisher's Exact Test and the Chi-Squared Test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Among the 80 samples from *H. pylori* positive patients included in the study, seven presented mutations in the *23S* rRNA region related to CLA resistance (8.7%). Of these, 85.7% (6/7) had mutations at position 2143, followed by 14.3% (1/7) at position 2142. Furthermore, in eighteen patients we identified mutations in the *gyrA* gene related to LVX resistance (22.5%). We observed that 83.3% (15/18) of the mutations in *gyrA* were at codon 87, while 16.7% (3/18) were at codon 91.

In addition, we investigated the association between *H. pylori*-positive patients with or without point mutations related to resistance to CLA or LVX with respect to sex, age, endoscopic diagnosis, previous treatment, drinking water source and the presence of CagA-EPIYA, as shown in Table 1. A statistically significant association was detected between mutations related to resistance with patients aged 45 years or more (p=0.024). Moreover, the absence of the pathogenicity factor CagA-EPIYA was observed in 68% (17/25) of resistant samples.

Table 1 - Profile of *H. pylori*-positive patients resistant and not resistant to Clarithromycin (CLA) or Levofloxacin (LVX)

		Not Resistant (n=55)	Resistant to CLA or LVX (n=25)	*P* value
Gender	Female (n=41)	52.7%(29/55)	48.0% (12/25)	0.695
	Male (n=39)	47.3%(26/55)	52.0% (13/25)	
Age	≤ 45 years (n=34)	50.9% (28/55)	24.0% (6/25)	0.024
	> 45 years (n=46)	49.1% (27/55)	76.0% (19/25)	
Endoscopic Diagnostic	Normal Mucosa (n=3)	3.6% (2/55)	4.0% (1/25)	0.819
	Gastritis (n=54)	65.5% (36/55)	72.0% (18/25)	
	Gastric Ulcer (n=23)	30.9% (17/55)	24.0% (6/25)	
Previous treatment for *H. pylori*	No (n=70)	89.1% (49/55)	84.0% (21/25)	0.165
	≤ 12 months (n=7)	5.5% (3/55)	16.0% (4/25)	
	> 12 months (n=3)	5.5% (3/55)	0% (0/25)	
Source of water to drink	Public tap (n=68)	81.8% (45/55)	92.0% (23/25)	0.237
	Mineral water (n=12)	18.2% (10/55)	8.0% (2/25)	
cagA-EPIYA	No (n=50)	60.0% (33/55)	68.0% (17/25)	0.790
	ABC (n=19)	25.5% (14/55)	20.0% (5/25)	
	ABCC/ABCCC (n=11)	14.5% (8/55)	12.0% (3/25)	

cagA EPIYA: cytotoxin associated gene A-Glu-Pro-Ile-Tyr-Ala

DISCUSSION

Although effective treatment regimens for *H. pylori* infections are available, the local antimicrobial resistance profile of strains should be considered. In the present study, 8.7% (7/80) of the samples presented a mutation in the *23S* rRNA region associated with CLA resistance, similar to the average ratio reported by other Brazilian studies, which reported values ranging from 7 to 27%[5].

The prolonged use of CLA in the treatment of other infections could be related to the selective pressure that results in the emergence of resistant microorganisms. The mutation 2143 (A→G) was detected in 85.7% (6/7) of samples, whereas only one strain had a mutation in the 2142 region (A→G). The predominance of the A→G mutation at sites 2142 and 2143 was suggested by Nash and Inderlied[10] to be a result of the greater stability on the conformational change within the ribosome than mutations from A→T or A→C[10,11]. The mutation at position 2143 (A→G) of the *23S* rRNA gene has also been predominantly detected in other studies[4,12]. However, a large geographic variation can be observed, and an uneven distribution is possible even within a single country, highlighting the importance of characterizing the regional profiles of strains to provide a correct therapy[13,14].

Regarding LVX, used as an alternative to conventional antimicrobials for the treatment of *H. pylori*, we detected the presence of mutations in the *gyrA* gene in 22.5% (18/80) of the patients and these mutations confer LVX resistance. These results are similar to the ones found in another study carried out in Brazil[14]. The mutations were detected at positions Asn87 83.3% (15/18) and Asp91 16.7% (3/18) in *gyrA*. The presence of mutations in these regions also has been previously reported in several countries, including Brazil[3,8,15].

Several factors may be associated with antimicrobial resistance in *H. pylori*[4]. In this study, a significant association was detected among mutations related to resistance in patients older than 45 years old (p=0.024). The high consumption of these antimicrobials to treat other infections could explain why resistance to CLA and LVX is higher in older individuals, given the cumulative likelihood of exposure to these antimicrobials[16].

While no significant association was observed between antimicrobial resistant and the absence of the *cagA* gene, 68% (17/25) of samples that were resistant to CLA or LVX were CagA-EPIYA-negative, whereas 20% (5/25) had an ABC genotype, considered to have low pathogenicity, and only 12% (3/25) carried the ABCC/ABCCC genotype[9].

Strains carrying the *cagA* gene develop an intense inflammatory response in the gastric epithelial cells and thus can proliferate more rapidly than *cagA*-negative strains. As antimicrobials are primarily active upon bacteria during the logarithmic phase of growth, *cagA*-positive strains are probably more susceptible to antimicrobial activity than *cagA*-negative strains[17].

Antimicrobial pressure can cause a loss of specific virulence factors (*cagA*) and lead to genetic rearrangements (loss or gain of EPIYA) that affect the impact of tyrosine phosphorylation, which can alter the pathogenic process of *H. pylori* in cases of treatment failure[18]. In this study, only four patients who were previously treated, presented therapeutic failure. Interestingly, all samples presented an endoscopic diagnosis of normal gastric mucosa or gastritis. In addition, these samples showed mutations associated with resistance to CLA or LVX, and 3 of 4 were *cagA*-negative. These data confirm the importance of *cagA* expression as a predictor of successful eradication, which was also reported by Russo *et al.*[19].

In conclusion, in the population analyzed from the extreme South of Brazil, we observed high rates of antimicrobial resistance related to specific mutations, highlighting the importance of evaluating the antimicrobial resistance in circulating strains. Moreover, this study suggests a complex interplay involving the absence of the *cagA* pathogenicity marker and antimicrobial resistance, contributing to a deeper knowledge of *H. pylori* infection and clinical approaches for its treatment.

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AUTHORS' CONTRIBUTION

Júlia Silveira Vianna collected data, performed the experiments and wrote the article. Ivy Bastos Ramis, Daniela Fernandes Ramos and Pedro Eduardo Almeida da Silva oriented and critically reviewed the article. Otávio Leite Gastal and Renato Azevedo da Silva collected the gastric samples. Carla Vitola Gonçalves performed the statistical analysis

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