ORIGINAL ARTICLE

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ABSTRACT

The increase of H. pylori resistance to clarithromycin is a concern. This study evaluated the prevalence of H. pylori’s primary resistance to clarithromycin and its association with virulence factors in adult dyspeptic patients and asymptomatic children. The gastric mucosa from patients (153 gastritis, 24 gastric cancer, 21 peptic ulcer) and gastric juice obtained by string test from 24 H. pylori and 23S rRNA positive asymptomatic children were included. The clarithromycin resistance was assessed by TaqMan RT-PCR 23S rRNA point mutations, A2142G and/or A2143G, and H. pylori virulence markers by PCR. Overall, the clarithromycin resistance was 14.4% (32/222), 14.2% in adults, and 12% in children, whereas origin, gender, and disease were not distinctive factors. The most prevalent point mutation was A2143G (62.5%). The point mutation was significantly less frequent in cagA-positive (11.4%) than in cagA-negative (23.6%) strains (p=0.03 OR = 0.4 95%CI = 0.19 - 0.91) as well as in cagE-positive (10.2%), cagE-negative (21.2%) (p=0.03 OR: 0.4 I.C:0.20-0.91). No difference was found in iceA or vacA alleles genotypes. Primary resistance to clarithromycin was lower than that reported in Southeast Brazil. The cagA and cagE positive H. pylori samples have few point mutations suggesting that individuals infected with virulent strains may be more susceptible to anti-H. pylori treatment.

KEYWORDS: H. pylori. Virulence factors. Clarithromycin-resistance.

INTRODUCTION

The Helicobacter pylori (H. pylori) infection causes chronic gastritis, peptic ulcer disease, gastric adenocarcinoma, and MALT (mucosa-associated lymphoid tissue) lymphoma.

The efficacy of H. pylori eradication rates following the standard first-line therapy that includes clarithromycin and metronidazole has been declining due to the increasing resistance to antimicrobial agents in several countries. The prevalence of clarithromycin resistance varies among different geographical regions, indicating that studies on this subject are required. Notably, it has been considered that resistance to clarithromycin has predicted treatment failure.

The main cause of clarithromycin therapeutic failure lies in key mutations of the ribosomal 23S subunit. Among the three main 23S rRNA point mutations, A2142G, A2146G, and A2147G, formerly described as A2142C, A2142G, and A2143G, are predicted to be responsible for more than 90% of clarithromycin resistance cases in Western countries.

ABSTRACT

Clarithromycin-resistant H. pylori primary strains and virulence genotypes in the Northeastern region of Brazil

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The main cause of clarithromycin therapeutic failure lies in key mutations of the ribosomal 23S subunit. Among the three main 23S rRNA point mutations, A2142G, A2146G, and A2147G, formerly described as A2142C, A2142G, and A2143G, are predicted to be responsible for more than 90% of clarithromycin resistance cases in Western countries.
PCR-based (polymerase chain reaction) molecular methods can detect the mutations, either in bacterial culture samples or in gastric mucosa fragments, in addition to stool samples and gastric juice obtained by less-invasive string test procedures. The molecular methods may have an important role in the prompt detection of resistance to clarithromycin in clinical practice. Several studies have demonstrated that the existence of mutations identified by molecular tests has a direct correlation with culture-based susceptibility testing.

Virulence factors of *Helicobacter pylori* have an important role in the pathogenicity of the bacteria. However, its influence on drug resistance has not been well outlined yet.

The main *H. pylori* virulence factors are encoded by the vacuolating cytokine-associated gene (*vacA*) and the cytotoxin-associated gene A (*cagA*). There are at least 4 variable regions in the *vacA*: in the signal region, s1 and s2 alleles, and in the middle region, m1 or m2 alleles. These variable regions show diverse levels of toxicity to host cells, with the *vacA* s1/m1 being the most cytotoxin producer, followed by s1/m2, and s2/m2 genotypes that induce little or no toxicity. The *cagA* and *cagE* are located in the *cag* Pathogenicity Island (PAI), which encodes a type IV secretion system. Patients colonized by strains carrying the *cagE* and *cagA* genes as well as the *vacA* s1/m1 alleles have a greater risk to develop severe gastrointestinal diseases.

Another virulence marker, the *iceA* (gene induced by contact with gastric epithelial cells), has two alleles, *iceA1*, and *iceA2*. In some regions, it has been reported that the *iceA1* genotype is associated with gastric disease.

Although several studies evaluate clarithromycin resistance in Brazil, there is no study in the Ceará State, the Northeast part of the country. It should be noted that the prevalence of *H. pylori* infection is high, the children are infected earlier by the bacterium and the infection is significantly associated with gastric cancer in this region. Furthermore, few studies evaluated the association of *H. pylori* virulence genotypes with clarithromycin resistance.

This study aimed to evaluate the prevalence of primary resistance of *H. pylori* to clarithromycin, using RT-PCR in adult patients with gastric disease as well as asymptomatic children from an urban community in the Northeast region of Brazil. We also investigated the association between the resistance profiles and a panel of *H. pylori* virulence genotypes.

**MATERIALS AND METHODS**

This study was performed in the tertiary care Hospital Universitario Walter Cantidio (HUWC, Federal University of Ceará) and an urban community, the Parque Universitario. The study protocol was approved by the Research Ethics Committee of HUWC, N° 628.750, and performed in accordance with the ethical standards set out in an appropriate version of the Declaration of Helsinki, as revised in Brazil 2013. The informed consent was obtained from all subjects and/or their legal guardians.

Patients undergoing upper gastrointestinal endoscopy to investigate dyspeptic symptoms were invited to participate. The diagnosis of gastric cancer was confirmed by histopathology according to the Classification of Lauren.

*H. pylori*-positive patients who had never been treated for *H. pylori* infection were included. Patients were excluded if they had taken proton-pump inhibitors or antibiotics during the 8 weeks before endoscopy. Patients with renal, hepatic, or hematological diseases, as well as pregnant women, were also excluded from the study. All patients answered a questionnaire about clinical symptoms, and demographic data such as age, gender, and residence area. Patients were recruited from August 2015 to August 2017.

The gastric mucosa fragments were obtained through endoscopy, and gastric juices by enterotest. All samples were processed to obtain DNA in order to perform molecular biology studies.

**Patients**

The calculation of the sample size was performed by using a formula for infinite populations (higher than 100,000) considering a confidence level of 95%, with a 5.0% of maximum error. Taking into account that the clarithromycin resistance rate is approximately 14.0% in the Northeast part of Brazil, it was necessary to include at least 185 samples.

In total, 300 *H. pylori*-positive samples were evaluated, 260 gastric mucosa fragments were obtained through endoscopy and 40 gastric juice samples were collected by enterotest. Of these, 222 (74.0%) were 23S rRNA positive; 76.1% (198/260) of the gastric mucosa, and 60.0% (24/40) of the gastric juice.

**Enterotest**

A gelatin capsule containing a 90 cm long absorbent cotton thread was swallowed along with a max of 200 mL of water. 20 cm of the string’s length was attached to the patient’s cheek. After an hour, the string was removed, and the proximal 30 cm was discarded. The segment of the thread with the gastric mucus impregnated with juice was washed with 5 ml of saline solution and then placed in sterile vials containing 3 ml of brain heart infusion broth.
The liquid in the flask was centrifuged at 13000 rpm for 10 min and processed for DNA extraction. The samples were stored at -70 °C. The method was improved and previously validated by our group.

Genomic DNA extraction

The H. pylori DNA was extracted from gastric mucosa and gastric juice samples, as well as from the bacterium isolated by culture. H. pylori DNA was obtained using the QIAamp DNA extraction kit (QIAgen®, Hilden, Germany) by following the manufacturer’s guidelines. The DNA samples were stored at -20 °C until used for gene amplification.

Detection of the virulence factors

The virulence markers were determined by PCR performed in a Thermal Cycler 2400 (Perkin Elmer Applied Biosystems, Foster City, California, USA). Briefly, the “Master mix” solution for gene amplification consisted of 5.5 μL of distilled H2O, 12.5 μL of the enzyme Taq-DNA polymerase (GoTaq green - Promega®, Madison, Wisconsin, USA), 1.0 μL of the “primers” (Carlsbad, California, USA) specific to each gene, starting from the 5’ to the 3’ termination, in both directions (Forward and Reverse Primer). The reactions occurred separately for each gene. In each microtube, 5.0 μL of the extracted DNA was added in specific 200 μL microtubes. The amplified fragments were developed on a 1% agarose gel stained with ethidium bromide. For the amplification of the 23S gene as well as cagA14,18, cagE19, vacAs1, vacAs2, vacAm1, vacAm28, and iceA1/iceA211 genotypes, primers were used as previously described in the literature.

Optimizing the amount of DNA per sample

The DNA was quantified by ultraviolet absorbance at 260 nm. To determine the ideal amount of DNA for the assay, serial dilutions between 10 ng and 50 ng were made to evaluate the best reading profile of the device. The amount of DNA for each sample was adjusted to 30 ng, based on the observations already made by previous studies20,21.

Characterization of positive and negative controls

The H. pylori 23S rRNA fragment used as positive and negative controls was obtained by PCR amplification of H. pylori DNA-extracted from homogenates of bacterial cultures (strains with and without clarithromycin resistance evaluated by phenotypic method) using Hp23-F (5-CCACAGCGAT GTG GTCTCAG-3) and Hp23-R (5-CTCCATAAGAGCCAAAGCC-3) as primers, according to conventional PCR assay.

Assay for RT-PCR and allele discrimination

The point mutations of H. pylori 23S rRNA (A2142G and A2143G) were performed by the real-time polymerase chain reaction (RT-PCR) reported by Oleastro et al.20.

The assays were performed by using a 7500 RT-PCR system® device (Applied Biosystems, inc. Foster City, CA, USA). The reactions were prepared using Universal PCR Master Mix kit (Applied Biosystems, inc. Foster City, California, USA) optimized for reactions with the TaqMan® assay probe containing the AmpliTaq Gold DNA polymerase. The preparation and storage of the materials were carried out according to the manufacturer’s guidelines, except for the final volume of each reaction optimized for 10 µL. The mixture prepared for RT-PCR consisted of 5.0 µL of Universal Master Mix plus 4.5 µL of DNA solution containing 30 ng and 0.5 µL of prepared assay containing: 36 µM (Forward and Reverse Primer, Table 1) and a concentration of 8 µM TaqMan MGB probes (FAM and VIC)21. After that, the reaction plates were centrifuged for 1 min at 4500 rpm. The amplification conditions for PCR were: preheating at 50 °C for 2 min, polymerase activation at 95 °C for 10 min and 40 denaturation cycles of 15 s at 94 °C, 1 min at 58 °C, and 1 min at 72 °C.

Table 1 - Primers and probes used for point mutations.

| Primers and probes used for point mutations | Concentration/Primer sequence |
|--------------------------------------------|------------------------------|
| 23SA2142G-2142F                            | TCAGTGAAATTTGATGGGCGGTGAAAAA |
| 23SA2142G-2142R                            | CAGTGCTAAGTTGATGAAGGTCCA     |
| VIC: AAGACGGAAAGAC                         | Wild DNA probe               |
| FAM: AAGACGGGAAGAC                        | Mutant DNA probe             |
| 23SA2143G-2143F                            | TCAGTGAAATTGATGGGCGGTGAAAAA |
| 23SA2143G-2143R                            | CAGTGCTAAGTTGATGAAGGTCCA     |
| VIC: AAGACGGAAAGAC                         | Wild DNA probe               |
| FAM: CAAGACGGAGAGAC                        | Mutant DNA probe             |
92 °C and 1 min at 60 °C for extension and annealing. The polypropylene reaction plates for 96 reactions (MicroAmp 96-Well plates, Applied Biosystems, Foster City, California, USA) were covered with adhesives for high temperature and alcohol-resistant optical microplates (Optical adhesive film, Applied Biosystems, Foster City, California, USA). In all steps, the samples were immersed in ice under little exposure to light. Each sample was evaluated in duplicate and only samples whose amplification differences did not exceed 0.8 cycles (Δ Cq ≤ 0.8) were considered for analysis\textsuperscript{22}. In all samples, negative and positive controls were performed. The increase in fluorescence of the FAM or VIC dye indicates a homozygous genotype (mutation or wild), while an increase in both signals indicates a heterozygous genotype\textsuperscript{23}.

**Statistical analysis**

The SPSS Statistics for Windows (version 21.0, IBM Corp, Armonk, New York, USA) was used to perform all statistical analyses. Chi-square and Fisher’s exact tests were used to determine the statistical significance of association between categorical variables, when applicable. The mean value and standard deviation were used to describe numeric variables. A p-value of less than 0.05 was considered statistically significant.

**RESULTS**

**Characteristics of the patients**

Overall, 222 \textit{H. pylori} 23S rRNA positive samples were evaluated, from 198 adult patients and 24 asymptomatic subjects residing in the community. The demographic and clinical data of the 198 adult patients are shown in Table 2. Of the 24 asymptomatic subjects living in the community, 50% (12/24) were females, with a mean age and standard deviation of 12.92 ± 3.04, ranging from 8 to 18 years old, all of them from the urban area.

**Prevalence of clarithromycin resistance**

Overall, the primary prevalence of \textit{H. pylori} clarithromycin resistance, based on the presence of 23S rRNA point mutations A2142G and/or A2143G, was 14.4% (32/222), being 14.6% (29/198) in adult patients and 12.5% (3/24) in children living in the community. The A2142G and A2143G mutations were observed in 37.5% (12/32) and in 62.5% (20/32) of the \textit{H. pylori}-positive samples, respectively. In 4 of 32 (12.5%) samples, a double point mutation, A2142G plus A2143G, was observed. Samples containing simultaneously susceptible wild and resistant mutant strains (heteroresistant samples) were observed in 19 among 32 (59.4%) samples.

The prevalence of resistant did not significantly differ between the origin of the patients (31.5% rural vs 23.3% urban area, p=0.17), adults and children (14.6% vs 12%, p=0.77) male and female (14% vs 18%, p=0.87), gastritis and peptic ulcer disease (12.5% vs 14.6%, p=0.29) as well as between gastritis and gastric cancer (12.5% vs 14.6%, p=0.86) (Table 3).

**\textit{H. pylori}-genotypes of virulence**

The \textit{cag}A was identified in 75.2% (167/222) of the samples. The prevalence of point mutation conferring clarithromycin resistance was significantly less frequent (p=0.03 OR = 0.4 95%CI = 0.19 - 0.91) in \textit{cag}A-positive (19/167 - 11.4%) than in \textit{cag}A-negative (3/55 - 23.6%) strains. The most prevalent point mutation was A2143G (20/32-62.5%) (9/167-5.4% of \textit{cag}A positive and 11/55-20% of \textit{cag}A negative strains).

The \textit{cag}E was observed in 137 of 222 samples (61.7%). Among them, 14 (10.2%) had a point mutation. Point mutations were observed in 18 of 85 (21.2%) \textit{cag}E-negative samples. The difference between \textit{cag}E-positive and \textit{cag}E-negative samples was statistically significant (p=0.03 OR: 0.4 I.C:0.20-0.91). A2143G was the most prevalent mutation, being present in 7 of the 14 \textit{cag}E-positive samples and in 13 of the 18 negative ones (Table 4).

The \textit{ice}A1 and \textit{ice}A2 alleles was 14.9% (33/222) and 69.8% (155/222), respectively. A higher prevalence of mutant strains was observed in \textit{ice}A1-negative (30/189, 15.8%) than in \textit{ice}A1-positive (2/33, 6.0%) samples, without statistical significance (p=0.13) (Table 4). In respect to \textit{ice}A2 genotype, no difference in the prevalence

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**Table 2 - Distribution of 198 adult patients by age, gender, origin and gastric diseases.**

| Age          | 47.95 ± 14.53 (19 to 89 years) |
|--------------|--------------------------------|
| Gender       |                                |
| Male         | 43.4% (86)                     |
| Female       | 56.5% (112)                    |
| Origin       |                                |
| Rural        | 60.6% (120)                    |
| Urban        | 39.40% (68)                    |
| Gastric disease |                            |
| Gastric Cancer | 12.1% (24)                  |
| Peptic Ulcer Disease | 10.6% (21)          |
| Gastritis    | 77.3% (153)                    |

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of the point mutation was observed between iceA2-positive (22/155-14.1%) and iceA2-negative (10/67-14.9%) samples (Table 4).

No significant difference was observed between the vacA genotypes and the point mutation conferring H. pylori resistance, regarding vacAs1-positive and negative samples, s1m1-positive and -negative genotypes and s2m2-positive and -negative genotypes (Table 5).

**DISCUSSION**

This study aimed to evaluate H. pylori resistance to clarithromycin by using RT-PCR in gastric mucosa samples from adult patients and gastric juice sample obtained by string test from asymptomatic children resident in the community of the Northeastern region of Brazil. Furthermore, we assessed the association of cagA, cagE, vacA and iceA1, iceA2 (N = 222).

**Table 3** - Susceptibility of H. pylori to clarithromycin using RT-PCR according to gender, origin and gastro duodenal diseases in 198 adults.

|                  | Resistance, n (%) | Susceptible, n (%) | p value |
|------------------|-------------------|--------------------|---------|
| **Gender**       |                   |                    |         |
| Male (86)        | 13 (15.1)         | 73 (84.8)          | 0.87    |
| Female (112)     | 16 (14.2)         | 96 (85.7)          |         |
| **Origin**       |                   |                    |         |
| Inland (113)     | 19 (16.8)         | 94 (83.2)          | 0.32    |
| Capital (85)     | 10 (11.8)         | 75 (88.2)          |         |
| **Gastric Cancer vs. Gastritis** |                   |                    |         |
| Gastric Cancer (24) | 3 (12.5)        | 21 (87.5)          | 0.86    |
| Gastritis (153)  | 25 (16.3)         | 128 (83.6)         |         |
| **Peptic ulcer vs. Gastritis** |                 |                    |         |
| Peptic ulcer (21) | 1 (4.8)          | 20 (95.2)          | 0.29    |
| Gastritis (153)  | 25 (16.3)         | 128 (83.6)         |         |

**Table 4** - Profile of point mutations in the 23S rRNA gene by the TaqMan RT-PCR method and association with H. pylori genotypes cagA, cagE, and iceA1, iceA2 (N = 222)

|                  | General Mutation | Mutation A2142G | Mutation A2143G | Double Mutation |
|------------------|------------------|-----------------|-----------------|-----------------|
| cagA + (167)     | 19 (11.4%)       | 7 (4.1%)        | 9 (5.4%)        | 3 (1.8%)        |
| cagA - (55)      | 13 (23.6%)       | 1 (1.8%)        | 11 (20%)        | 1 (1.8%)        |
| p value          | 0.025            | 0.413           | 0.003           | 0.992           |
| OR (IC 95%)      | 0.7 (0.567-1.025) | -               | -               | -               |
| cagE + (137)     | 14 (10.2%)       | 4 (2.9%)        | 7 (5.1%)        | 3 (2.1%)        |
| cagE - (85)      | 18 (21.2%)       | 4 (4.7%)        | 13 (15.2%)      | 1 (1.1%)        |
| p value          | 0.03             | 0.754           | 0.021           | 0.968           |
| OR (IC 95%)      | 0.4 (0.20-1.91)  | -               | -               | -               |
| IceA1+ (33)      | 2 (6.0%)         | 0 (0%)          | 2 (6.0%)        | 0 (0%)          |
| IceA1- (189)     | 30 (15.8%)       | 8 (4.2%)        | 18 (9.5%)       | 4 (2.1%)        |
| p value          | 0.139            | 0.485           | 0.755           | 0.893           |
| IceA2+ (155)     | 22 (14.1%)       | 5 (3.2%)        | 13 (8.3%)       | 4 (2.5%)        |
| IceA2- (67)      | 10 (14.9%)       | 4 (5.4%)        | 7 (10.4%)       | 0 (0%)          |
| p value          | 0.887            | 0.646           | 0.813           | 0.437           |

This study aimed to evaluate H. pylori resistance to clarithromycin by using RT-PCR in gastric mucosa samples from adult patients and gastric juice sample obtained by string test from asymptomatic children resident in the community of the Northeastern region of Brazil.
The overall prevalence of *H. pylori* clarithromycin resistance in this study was 14.4%, a ratio that is acceptable for the empirical use of clarithromycin-based regimens. Several studies have used genotypic methods to detect primary clarithromycin resistance in Brazil, without major variations among them. A National study that evaluated samples from four different regions using GenoType HelicoDR showed an overall prevalence of 16.9%. A primary resistance rate of 14.5% using phenotypic and genotypic methods was reported in the Northeast and 16.6% in the North of Brazil.

In our cohort, the resistance to clarithromycin was more prevalent in patients with gastritis when compared to patients with peptic ulcer or gastric cancer, but without statistical significance, which may be explained by the low number of patients with peptic ulcer. On the other hand, a study from Turkey found a higher prevalence of clarithromycin resistance in the patients with peptic ulcer 40.9% when compared to those with gastritis. 24.6%.

This study was the first to evaluate the resistance to clarithromycin in gastric juice from asymptomatic children, which showed no difference between adults and children with a lower prevalence when compared to those reported in studies conducted in São Paulo, the Southeast region of Brazil, where a resistance rate of 27% was found in samples from children and adolescents who underwent endoscopy due to gastrointestinal symptoms in 2010, 26.7% in 2011, and 19.5% in 2014. A higher prevalence of resistance to clarithromycin in children has been attributed to the greater use of antibiotics in this age group. It is possible that the lower prevalence found in our study is due to the fact that we evaluated asymptomatic children from a low-income community.

Among the resistant strains, the A2143G mutation was the most frequently observed, in accordance with researches from Europe, Japan, Spain, Australia, France and Brazil. Conversely, studies from Egypt reported that the A2142G mutation was more frequent than the A2143G.

Heteroresistance to clarithromycin (samples that have a simultaneous presence of wild and mutant strains) were

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**Table 5 - Profile of point mutations in the 23s rRNA gene by the RT-PCR method and association with vacA alleles (N = 222).**

| General Mutation | Mutation A2142G | Mutation A2143G | Double Mutation |
|------------------|-----------------|-----------------|-----------------|
| VacAs1+(173)     | 24 (13.8%)      | 7 (4.0%)        | 13 (7.5%)       | 4 (2.3%)       |
| VacAs1+(49)      | 8 (16.3%)       | 1 (2.0%)        | 7 (14.2%)       | 0 (0%)         |
| p value          | 0.67            |                 |                 |                |
| VacAs2+(26)      | 4 (15.3%)       | 0 (0%)          | 4(15.3%)        | 0 (0%)         |
| VacAs2+(196)     | 28 (14.2%)      | 8 (4.0%)        | 1 (8.1%)        | 2 (2.0%)       |
| p value          | 0.99            |                 |                 |                |
| VacAm1+(178)     | 25 (14.0%)      | 6 (3.3%)        | 15 (8.4%)       | 4 (2.2%)       |
| VacAm1-(44)      | 7 (15.9%)       | 2 (4.5%)        | 5 (11.3%)       | 0 (0%)         |
| p value          | 0.94            |                 |                 |                |
| VacAm2+(42)      | 5 (11.9%)       | 0 (0%)          | 5(11.9%)        | 0 (0%)         |
| VacAm2-(180)     | 27 (15.0%)      | 8 (4.5%)        | 15 (8.3%)       | 0 (0%)         |
| p value          | 0.79            |                 |                 |                |
| VacAs1m1 +(145)  | 19 (13.1%)      | 6 (4.1%)        | 9 (6.2%)        | 4 (2.7%)       |
| VacAs1m1 -(77)   | 13 (16.8%)      | 2 (2.6%)        | 11 (14.2%)      | 0 (0%)         |
| p value          | 0.57            |                 |                 |                |
| VacAs1m2+(30)    | 4 (13.3%)       | 0 (0%)          | 4(13.3%)        | 0 (0%)         |
| VacAs1m2 -(192)  | 28 (14.5%)      | 8 (4.1%)        | 16 (8.3%)       | 4 (2.0%)       |
| p value          | 0.99            |                 |                 |                |
| VacAs2m1 +(20)   | 3 (15.0%)       | 1 (5.0%)        | 1 (5.0%)        | 5 (5.0%)       |
| VacAs2m1 -(202)  | 29 (14.4%)      | 7 (3.5%)        | 19 (9.4%)       | 3 (2.6%)       |
| p value          | 0.99            |                 |                 |                |
| VacAs2m2+(18)    | 3 (16.6%)       | 0               | 3 (16.6%)       | 0              |
| VacAs2m2 -(204)  | 29 (14.2%)      | 8 (3.9%)        | 17 (8.3%)       | 4 (2.0%)       |
| p value          | 0.99            |                 |                 |                |
observed in 59.4% of our samples, a finding that has also been reported in other studies in Brazil, such as in Recife (77.8%)\textsuperscript{17} and in a national multi-center study (73.5%)\textsuperscript{16}. High rates of clarithromycin heteroresistance were also detected in samples from Spain (51.3%)\textsuperscript{33} and Italy (76.3%)\textsuperscript{34}. The association between \textit{H. pylori} virulence factors and clarithromycin resistance has been evaluated in several researches with divergent results\textsuperscript{35}. In this study, the overall prevalence of \textit{cagA} positivity was 75.5%, 23S rRNA point mutation of clarithromycin was found in 11% of these strains with a significant association with \textit{cagA}-negative strains. The low prevalence of primary resistance to clarithromycin in \textit{H. pylori} \textit{cagA}-positive strains coincides with studies reported from the Southeast region of Brazil\textsuperscript{36}. On the contrary, other studies demonstrated that \textit{cagA} positivity was not associated with resistance status\textsuperscript{31,32,37}. A meta-analysis study showed that the treatment efficacy was lower in patients infected by \textit{cagA}-negative and \textit{vacA}s2-positive strains and concluded that the treatment success is higher in subjects colonized with highly virulent strains\textsuperscript{38}. It has been postulated that infection with \textit{cagA}-positive strains, a more virulent genotype, is associated with high gastric epithelium lesions, which may facilitate the antimicrobials to reach higher concentrations in inflamed mucosa\textsuperscript{38}. In this study, the absence of \textit{cagE} gene was significantly associated with the resistance of clarithromycin. Conversely, a study from Turkey reported that the presence of \textit{cagE} gene was associated with clarithromycin resistance\textsuperscript{39} while other studies, such as that the one reported by Godoy \textit{et al.}\textsuperscript{36} in the Southeast region of Brazil, have demonstrated no association. These differences among regions point that further evaluation is needed.

Regarding the \textit{iceA}, our results showed a high prevalence of \textit{iceA}2-positive strains, in accordance with the study of Yamaoka \textit{et al.}\textsuperscript{40} which demonstrated a higher prevalence of \textit{iceA}1 than \textit{iceA}2 strains in Asian countries, while the opposite was observed in the Western countries. Similarly to that observed in the Southeast region of Brazil\textsuperscript{36} we did not observe an association between \textit{iceA} genotypes and clarithromycin point mutations.

This study has several limitations; first we did not compare clarithromycin resistance by using RT-PCR with phenotypic methods; however, the accuracy of RT-PCR to detect clarithromycin resistance has been widely evaluated in other studies. In addition, RT-PCR was properly standardized using \textit{H. pylori} strains resistant and sensitive to clarithromycin by phenotypic method. Second, the number of included samples of children is small, which may be explained by the fact that we studied asymptomatic children from a specific community.

**CONCLUSION**

In conclusion, the \textit{H. pylori} resistance rate to clarithromycin we observed (14.41%) is an acceptable rate to empirically use the standard scheme for anti-\textit{H. pylori} containing clarithromycin. The \textit{H. pylori} \textit{cagA} and \textit{cagE} positive-samples have few punctual mutations that confer resistance to clarithromycin. Possibly the subjects infected with virulent strains are more susceptible to anti-\textit{H. pylori} treatment containing clarithromycin.

**ACKNOWLEDGMENTS**

This work was supported by Fundacao de Apoio ao Desenvolvimento Cientifico e Tecnologico do Ceara, grant Nº 13506715-4. We wish to thank Antonio B. Viana Junior for the statistical analysis.

**AUTHORS’ CONTRIBUTIONS**

LLBCB and TGSB conceived and designed the study; OGRA, FSM and ELO contributed to data collection; HLRJ, RTO, TGSB and RPF did the molecular genetic analysis; TGSB wrote the draft of the manuscript; LLBCB and DMMQ revised the manuscript. All authors contributed to the interpretation of data and approved the final manuscript.

**CONFLICT OF INTERESTS**

The authors declare that they have no conflict of interests.

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