

Characterization of 23S rRNA domain V mutations in gastric biopsy patients from the eastern Amazon

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Resistance of Helicobacter pylori to clarithromycin is characterised by simple point mutations in the 23S ribosomal RNA (rRNA) gene and is responsible for the majority of cases of failure to eradicate this bacterium. In this paper, we characterised the variability of the 23S rRNA gene in biopsies of patients with gastric pathologies in the eastern Amazon (Northern Region of Brazil) using PCR and sequencing. A total of 49 sequences of H. pylori strains were analysed and of those, 75.6% presented nucleotide substitutions: A2142G (3.3%), T2182C (12.9%), G2224A (6.45%), T2215C (61.3%), A2192G (3.3%), G2204C (6.4%) and T2221C (6.4%). Of the mutations identified, four are known mutations related to cases of resistance and 16.1% are not yet described, revealing a high prevalence of mutations in the H. pylori 23S rRNA gene among the strains circulating in the in the eastern Amazon. The high prevalence in individuals with gastric pathologies in the Northern Region of Brazil demonstrates the need for characterising the profile of these strains to provide correct therapy for patients, considering that mutations in this gene are normally associated with resistance to the primary medication used in controlling H. pylori infection.

Key words: *Helicobacter pylori* - 23S rRNA gene - clarithromycin - resistance - mutation

Helicobacter pylori is a microorganism associated with gastric pathologies, such as peptic ulcers, chronic gastritis and stomach cancer (Dunn et al. 1997). Treatment consists of a triple therapy that combines a protein inhibitor with antibiotics, with clarithromycin being the first choice (Asaka et al. 2001, Ribeiro et al. 2003) because it is acid-stable and easily absorbed in the gastric mucosa and, therefore, effective against *H. pylori* (Megraud 2004, Yilmaz & Demiray 2007). Thus, resistance by this bacterium to the antibiotic is considered to be one of the major reasons for treatment failure (Yilmaz & Demiray 2007).

Clarithromycin is a macrolide that has the ability to inhibit protein synthesis in *H. pylori* because of its interaction with ribosomal subunit 50S in the microorganism (Versalovic et al. 1996, Garrido & Toledo 2007). The mechanism of action for clarithromycin is to bind to the peptidyltransferase loop in domain V of the 23S ribosomal RNA (rRNA) gene, which interferes with protein elongation, efficiently blocking bacterial protein synthesis (Yilmaz & Demiray 2007).

It is believed that resistance to clarithromycin is a result of structural changes in the 23S rRNA molecule that develop when a nucleotide substitution occurs near this ribosome binding site, impeding the drug from binding and reducing its efficacy (Graham & Qureshi

2000). Mutations in domain V of the 23S rRNA gene have been associated with an important role in resistance to clarithromycin (Versalovic et al. 1996, 1997, Umegaki et al. 2000, Rimbara et al. 2007). These substitutions result in reduced ribosomal affinity for various macrolides, leading to resistance (Umegaki et al. 2000, Rimbara et al. 2007). Thus, there is a failure in eradicating the bacteria from the gastrointestinal tract, which complicates treatment of gastroduodenal diseases with which the infection is associated (Rimbara et al. 2007).

The most prevalent and well-documented mutations in the 23S rRNA gene occur in two adjacent nucleotide positions, 2142 and 2143, and include an adenine-guanine transition at position 2142 (A2142G) or 2143 (A2143G) and an adenine-cytosine transversion at position 2142 (A2142C) (Versalovic et al. 1996, 1997, Occhinalli et al. 1997). Other mutations that are associated with resistance have occasionally been observed in region V of the 23S rRNA gene, including G1939A, A2115G, G2141A, A2144G, C2147G, T2182C, G2224A and T2215C (Stone et al. 1996, Taylor et al. 1997, Song et al. 2000, Kim et al. 2002, Hao et al. 2004, Garrido & Toledo 2007). The number of mutations in this region indicates that any alteration in this domain of the gene may be related to bacterial resistance to clarithromycin.

The state of Pará (PA), in the Brazilian Amazon, has a high prevalence of *H. pylori* infection in patients with gastric pathologies (Aguar et al. 2002, Martins et al. 2005, Melo-Barbosa et al. 2009). Because of a recent increase in failure of the treatment scheme for the bacteria due to clarithromycin resistance (Magalhães et al. 2002, Hao et al. 2004, Kim et al. 2008), the objective of this paper was to demonstrate the modifications in the *H. pylori* 23S rRNA gene isolated from eastern Amazon patients using a metagenomic approach.

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PATIENTS, MATERIALS AND METHODS

Patients - In 2007 and 2008, gastric biopsy samples were collected from patients receiving upper digestive endoscopy at the João de Barros Barreto and Ophir Loyola in the city of Belém, PA, in the Northern Region of Brazil. All patients were informed of the objective of the research and their participation was requested through a free and informed consent agreement authorising collection of samples for the study. This study was approved by the Tropical Medicine Ethical Committee at Universidade Federal do Pará. The patients submitted to the study have been diagnosed with gastritis, peptic ulcer or gastric cancer. No patient had been treated for *H. pylori* infection.

DNA isolation and PCR amplification - DNA from the samples was extracted using the phenol-chloroform method (Sambrook et al. 1989). A sample pool was created composed of the DNA from individuals known to be infected by the bacteria ($n = 250$). Amplification of DNA from patient samples and the sample pool was performed using primers 5'-CCACAGCGATGTGGTCTCAG-3' (position 1820-1839) and 5'-CTCCATAAGAGCCT-GACT-3' (position 2244-2225), which amplified a 425-bp fragment from region V of the *H. pylori* 23S rRNA gene (Occhinali et al. 1997). The 50 μ L reaction contained 75 mM Tris-HCl, 20 mM KCl pH 8.4, 1.5 mM MgCl₂, 0.2 mM deoxynucleotides, 1 μ M each primer, 2 U Platinum Taq DNA polymerase (Invitrogen, Life Technologies) and 100 ng genomic DNA. The cycling program corresponded to 1 cycle at 95°C for 5 min, 35 cycles at 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec and a final extension cycle at 72°C for 10 min. The amplicon of 425 bp was visualised on a 2% agarose gel.

Genomic sequencing - After amplification, the fragment was excised from the agarose gel, purified with the GE GFX kit, inserted into a pGEM[®]-T Easy Vector System (Promega Corp, USA) following the manufacturer's instructions and then transformed by electroporation into TOP 10 *Escherichia coli*. The transformed bacteria were grown on plates using 2XYT culture media containing ampicilin (EMS, Brazil) and X-gal (GE Health Care, USA). The recombinant clones were selected and grown in deep well plates with TARTOFF broth containing ampicilin. Recombinant clones were isolated using a miniprep and then sequenced in a MegaBACE1000 automatic isolator (GE).

Sequence analysis - The resulting sequences were aligned and manually edited with the ClustalW application using the Bioedit sequence alignment editor 7.0.0 - BioEdit software (Hall 1999), with GenBank accession sequence U27270 as the reference.

RESULTS

Sequencing of the domain V fragment of the 23S rRNA gene of *H. pylori* resulted in 49 reads, which were aligned and edited using the Bioedit application. Chimeric sequences were detected and eliminated from the database and the remaining 41 sequences were analysed for diversity.

The domain V mutations of the 23S rRNA gene were determined by comparison with the reference strain (UA802) and we verified that 75.6% (31/41) of the obtained reads presented some type of nucleotide substitution in the sequence.

Among the observed substitutions, we found seven different types of mutations in different reads (Table), including four that have been described in the literature as being associated with resistance to clarithromycin (A2143G, T2182C, T2215C and G2224). Additionally, we found three novel substitutions not characterised in any previously described population: G2204C, A2192G and T2221C. None of the sequences had more than one nucleotide change.

The substitutions encountered in the sequencing reads occurred with the following prevalence: A2143G (3.3%), T2182C (12.9%), G2224A (6.45%), T2215C (61.3%), A2192G (3.3%), G2204C (6.4%) and T2221C (6.4%).

The seven sequences found in this study were submitted to GenBank, generating accession GQ395614, GQ395615, GQ395616, GQ395617, GQ395618, GQ395619 and GQ395620 (Table).

DISCUSSION

The prevalence of mutations found in domain V of the 23S rRNA gene of *H. pylori* in patients from the Northern Region of Brazil was high; 76.5% of the obtained sequences presented some type of nucleotide substitution. According to prior studies, alterations in this domain of the gene confer bacterial resistance to clarithromycin (Versalovic et al. 1996, Occhialini et al. 1997, Van Doorn et al. 2001, Posteraro et al. 2006, Kim et al. 2008).

It is believed that substitutions found in this *H. pylori* gene may be linked to the growing use of this antibiotic not only in eradicating *H. pylori* but also in treating respiratory tract infections (Megraud 1998, Ribeiro et al. 2003, Posteraro et al. 2006), which leads to antibiotic resistant strains. Furthermore, this bacterial species has extensive rearrangements within its genome (Dunn et al. 1997, Covacci et al. 1999, Velázquez & Feirtag 1999) used for adapting to hosts (Suerbaum & Josenhans 2008), mainly leading to development of resistance to antibiotics like clarithromycin.

Studies have demonstrated that the frequency of resistance to this antibiotic has varied around the world according to the geographic region and subgroups within populations (Graham 1998, Kim et al. 2008) and in recent years, a significant increase in the rates of *H. pylori* strains resistant to clarithromycin has been documented (Magalhães et al. 2002, Posteraro et al. 2006). In Europe, resistance rates are 2-24%, while they are 13-18% in Japan and 12-25% in North America (Nakamura et al. 2007). Kim et al. (2008) demonstrated an increase in cases of resistant strains reaching 85.1% in Korea that was correlated with the reduction in bacteria in individuals in that country.

In Southeastern Brazil, Magalhães et al. (2002) found a 9.85% prevalence of resistant strains and in the following year, Godoy et al. (2003) described an increase to 16%. In this study, the Northern Region has a much higher rate than the average observed in the Southeastern Brazilian population, presenting four of the known mutations

TABLE
Nucleotide substitutions verified in domain V of the 23S gene of rRNA in *Helicobacter pylori* found in patients with gastric pathologies in the Northern Region of Brazil (2007-2008)

Clone name	Mutation ^a	Frequency	GenBank access	Reference
HPPAA09	G2204C	2	GQ395614	This study
HPPAA11	A2192G	1	GQ395615	This study
HPPAB09	T2221C	2	GQ395616	This study
HPPAH12	A2143G	1	GQ395617	Versalovic et al. (1996)
HPPAE09	T2215C	19	GQ395618	Song et al. (2000)
HPPAD10	G2224A	2	GQ395619	Hao et al. (2004)
HPPAA12	T2182C	4	GQ395620	Kim et al. (2002)

a: the structure and definition of positions is in agreement with what was proposed by Taylor et al. (1997).

related to clarithromycin resistance, which may be related to heterogeneity in the *H. pylori* strains that colonise the different regions of Brazil (Kodaira et al. 2000).

Four known mutations were detected in this study, A2143G (Versalovic et al. 1996), T2182C (Kim et al. 2002), G2224A (Hao et al. 2004) and T2215C (Song et al. 2000), with the latter being quite prevalent (62.95%). Also, three novel mutations were identified and characterised in bacteria within the gastrointestinal metabiome. Additionally, because these mutations are in the domain of the gene normally related to clarithromycin resistance (Versalovic et al. 1996, Occhialini et al. 1997, Ribeiro et al. 2003), new studies must be performed using culture-dependent approaches to confirm association of bacterial antibiotic resistance with the novel nucleotide substitutions in domain V of the 23S rRNA gene detected in this study.

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