

CLARITHROMYCIN-RESISTANT *Helicobacter pylori* IN RECIFE, BRAZIL, DIRECTLY IDENTIFIED FROM GASTRIC BIOPSIES BY POLYMERASE CHAIN REACTION

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ABSTRACT – *Context* - Clarithromycin is the most effective drug used in the eradication of infection by *Helicobacter pylori*. Due to worldwide increase in resistance, pre-treatment susceptibility testing for clarithromycin is recommended. *Objectives* - To evaluate the prevalence of clarithromycin resistance of *H. pylori* in Recife, a city in Northeast Brazil. *Methods* - From January 2006 to December 2007, 114 gastric biopsy samples positive for *H. pylori* at culture were directly assayed by polymerase chain reaction (PCR) to detect the most frequent point mutations involved in clarithromycin resistance. Results were compared with those obtained by Etests. *Results* - Molecular and phenotypic methods showed 111 (97.4%) susceptible or resistant concordant results. PCR detected 3 (2.6%) biopsy specimens with *H. pylori*-resistant genotypes, which were misdiagnosed as susceptible by Etests. In Recife, based on PCR results, primary clarithromycin resistance was found in 15 (16.5%) patients, prevalence close to that observed in Southeast Brazil. Resistance increased to 52% among previously treated patients. The point mutation A2143G was present in 20 (71.4%) of specimens and A2142G, in 8 (28.6%) of specimens. A2142C was not found. *Conclusion* - In Recife, the prevalence of primary clarithromycin resistance, 16.5%, showed the need for pretreatment susceptibility testing in *H. pylori* infections.

HEADINGS - *Helicobacter pylori*. Helicobacter infections. Clarithromycin. Drug resistance, microbial. Polymerase chain reaction.

INTRODUCTION

The worldwide increase in antibiotic resistance to infection by *H. pylori* necessitates that therapy be devised on the basis of the testing of antibiotic susceptibility⁽⁴⁾. This is advocated particularly for clarithromycin, the most effective drug used in the eradication of the organism, resistance to which is the main factor responsible for treatment failure^(3, 7, 9).

Pretreatment susceptibility testing for clarithromycin is needed if the prevalence of primary resistance in the community reaches 15%-20%⁽⁸⁾. The most frequent point mutations responsible for clarithromycin resistance, A2142G, A2143G, and A2142C, are adjacent within the *H. pylori* 23S rRNA gene^(10, 19), allowing diverse molecular methods for their identification⁽¹⁰⁾. Quantitative real-time polymerase chain reaction (qPCR) is a simple assay routinely used for multiple diagnostic purposes. It permits identification of the specific point mutation involved in clarithromycin resistance, as well as quantification of *H. pylori* populations in gastric samples^(6, 10).

The aims of this study were to determine the prevalence of clarithromycin resistance in Recife, a city in Northeast Brazil; identify the point mutations responsible for this resistance; and to compare the TaqMan[®] qPCR assay⁽²⁾ directly applied to gastric biopsies with matched isolates examined by Etest.

METHODS

Gastric biopsies and culturing

From January 2006 to December 2007, 494 adult dyspeptic patients were referred to a private medical center in Recife, Brazil, for diagnostic endoscopy. Three gastric biopsies (two from the antrum, one from the fundus) were taken and immersed together into micro-tubes containing a transport medium, 0.5 mL of brain heart infusion (BHI) broth (Becton Dickinson, Cockeysville, MD, USA) with 0.5% agar. Biopsies were done after obtaining informed consent in accordance with the Declaration of Helsinki. Gastric samples were homogenized, and one aliquot cultured on brucella agar (Becton Dickinson) with 10% inactivated fetal calf

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serum and a mixture of antibiotics: polymyxin B 5000 IU/L, vancomycin 10 mg/L, trimethoprim 5 mg/L, and amphotericin B 5 mg/L (Sigma, St Louis, MO, USA). Plates were incubated at 37°C for 7 days under micro-aerobic conditions by using the Campy-Pak system (Becton Dickinson). The rest of the homogenate was stored at -80°C for subsequent PCR tests.

We examined the first 114 consecutive gastric samples (mean age, 42 years, 62% females) positive for *H. pylori* at culture. Of these, 23 (20.2%) came from patients who had undergone at least one previous treatment with clarithromycin, amoxicillin and omeprazole for eradicating the organism.

Etest assay

From the isolation medium, *H. pylori* colonies were subcultured onto Muller-Hinton agar plates (Becton Dickinson) with 10% defibrinated sheep blood (SBA). After 48 h in the conditions stated above, bacterial growth was suspended in BHI broth and adjusted to a density equal to a McFarland-4 standard. Cotton swabs were dipped into bacterial suspensions and evenly spread onto pre-dried SBA plates. Clarithromycin strips (AB Biodisk, Solna, Sweden) were applied onto the surface. Plates were incubated in micro-aerobic jars and examined after 72 h. Minimum inhibitory concentrations (MIC) were recorded as the point at which the ellipse intersected the interpretive scale on the strip. Cultures showing MIC ≥ 1 mg/L were considered resistant.

TaqMan® qPCR

Biopsy homogenates were defrosted and DNA extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions.

qPCR assays were directed for detecting four alleles in domain V of the 23S rRNA gene, the wild-type, and the point mutations A2142G, A2143G, and A2142C, associated with macrolide resistance in *H. pylori*^(10, 19). For molecular tests, we used an identical TaqMan® PCR technique, with probes and primers exactly as previously designed⁽²⁾. Primers and probes, synthesized by Applied Biosystems, Incorporated, Foster City, CA, USA, were as follows:

Primer 23S-F: TCAGTGAAATTGTAGTGGAGGTGAAAA.

Primer 23S-R: CAGTGCTAAGTTGTAGTAAAGGTCCA.

TaqMan® probe 23S-WT-TET: AAGACGGAAAGACC for DNA wild-type.

TaqMan® probe 23S-A2142G-PFAM: AAGACGGGAAGACC for mutated DNA.

TaqMan® probe 23S-A2143G-PFAM: CAAGACGGAGAGACC for mutated DNA.

TaqMan® probe 23S-A2142C-PFAM: AGACGGCAAGACC for mutated DNA.

qPCR assays were done in three vessels, each containing a probe directed to a different point mutation and the probe targeting the wild genotype, a duplex model.

Positive and negative controls were included in each assay. Light emission was analyzed in an iCycler iQ5, real-time detection system, software version 3.1 (Bio-Rad, Hercules, CA, USA). Threshold cycles (C_T) were determined by the second derivative function as the number of cycles at which

fluorescence exceeded the threshold limit. Tenfold serial dilutions of *H. pylori* DNA (10 ng to 10 fg) were used as a DNA template to determine the detection limit of the 23S qPCR assay. qPCR and Etests showing discrepant results were re-tested in triplicate.

Statistical analyses

Numerical values were shown as medians with ranges. Statistical differences between the genotypes A2142G and A2143G, and MICs were calculated by Fisher's exact test. $P < 0.05$ was considered statistically significant.

RESULTS

Of 114 biopsies examined, qPCR and Etest showed 111 (97.4%) susceptible or resistant concordant results. qPCR detected a mixture of mutant and wild genotypes in three (2.6%) other gastric samples, which were misdiagnosed by Etests as only carrying susceptible organisms (Figure 1).

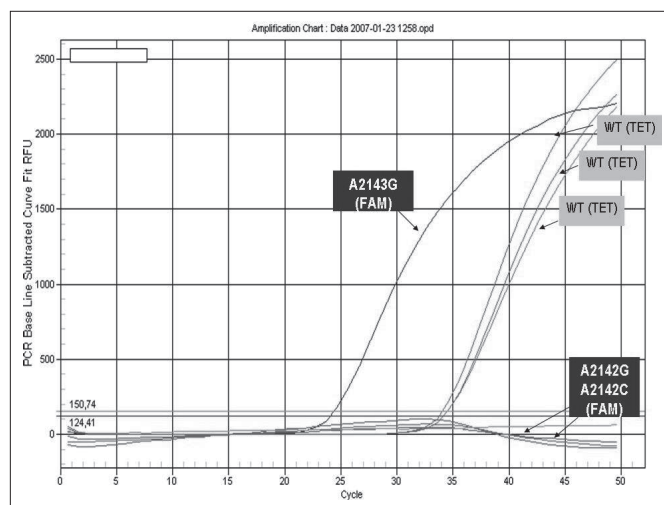


FIGURE 1. Amplification of DNA extracted from a gastric biopsy sample naturally infected with a mixture of susceptible, 10^2 fg/PCR and resistant 10^3 fg/PCR *H. pylori* populations. The mutant allele A2143G (FAM) predominates on the wild genotype WT (TET)

qPCR assays could consistently detect 45 fg of pure *H. pylori* DNA per reaction. Detection threshold was 25 bacterial cells, $C_T = 37$, because one bacterium corresponds to 1.8 fg of DNA (1,667,867 bp) per genome⁽¹⁸⁾. It was defined as the cutoff because results were reproducible at this C_T value. Except one biopsy sample which presented a C_T value of 37 cycles, all the others showed C_T values of < 37 independently of the number of colonies found on the isolating medium plates. The qPCR assay therefore showed a sensitivity of 100% relative to culture. C_T values were 37–15 (median = 26) due to variations in the density of *H. pylori* gastric colonization.

Of 27 gastric samples showing *H. pylori* with resistant genotypes, 21 (77.8%) were also infected with strains carrying the wild-type allele.

Based on qPCR results, the prevalence of the primary clarithromycin resistance in Recife was 16.5%.

Concerning resistant genotypes, A2143G, present in 20 (71.4%) of the resistant strains, predominated in relation to the genotype A2142G, present in 8 (28.6%) of the resistant strains. The point mutation A2142C was not found. Correlation between genotypes and MIC levels was not observed ($P > 0.05$); A2142G and A2143G showed the same MIC level, 12 µg/mL (Table 1).

TABLE 1. Genotypes of *H. pylori* 23S rRNA, directly identified by PCR in gastric biopsies, related to clarithromycin MIC rates obtained by Etesting shared isolates (n = 114)

Genotype of 23S rRNA	n	Etest MIC (µg/mL)	
		Median	Range
WT	87	0.023	<0.016–0.64
A2142G single	4	14	12 ≥256
A2143G single	2	22	12–32
Mixed WT and A2142G	3	128	0.064 ¹ –256
Mixed WT and A2143G	18	10	0.064 ² –256

¹One susceptible by Etest; ²Two susceptible by Etest; WT = Wild-type

DISCUSSION

Present results indicate that the PCR platform used in this work presented highly efficient amplifications, that is, a slope of 3.2 was obtained with the standard curve from 10 ng to 10 fg, and linear regression analysis showed $R^2 = 1$. Therefore, real-time PCR would be the preferential assay for detecting clarithromycin resistance in *H. pylori*. Moreover, qPCR is able to detect a mixture of mutant and wild genotypes. In present series, for instance, three (2.6%) gastric samples, which were misdiagnosed by Etests as only carrying susceptible organisms, showed at PCR resistant populations (Figure 1).

On Etest, one of these false-susceptible isolates grew several colonies within the inhibition ellipse. DNA extracted from these colonies, submitted to qPCR, showed an identical *H. pylori* genotype as that seen in direct qPCR assay. Mutant colonies were not seen in Etest ellipses of the two discrepant samples. Mutations sometimes involve a biologic cost; and bacterial fitness can be affected negatively. Strains of *Escherichia coli*, which are furazolidone-resistant, grow at a lower rate than the susceptible isogenic parent when cultured without the drug⁽¹⁶⁾. Clarithromycin-resistant strains of *H. pylori* are defective in

functions required for colonizing the mouse stomach⁽¹⁾. On the same culture media, mutant cells would be surpassed by susceptible cells, producing a misleading bacterial inoculum with a higher ratio of the wild genotype. In such situations, only an appropriate molecular method can discern a few *H. pylori* cells carrying resistant alleles^(11, 13).

Gastric samples showing *H. pylori* with resistant genotypes, 21 (77.8%) were also infected with strains carrying the wild-type allele, a high number of biopsies with mixed populations in comparison with other studies^(11, 12, 13, 17, 20). This was probably due to the design of the qPCR assay. First, it was built with TaqMan[®] minor groove binding (MGB) probes⁽²⁾. The efficiency of MGB probes for distinguishing a single-base mismatch on DNA targets has been established^(5, 20). Second, a different vessel was used for amplifying each of the three point mutations investigated. The wild-type gene could be amplified alone in two of the three vessels (i.e., without competition from an occasional resistant allele). When DNA templates derived from resistant and susceptible *H. pylori* strains are simultaneously assayed by PCR in the same vessel, the under-represented allele would have its amplification partly harmed^(11, 17). PCR formats, based on analysis of post-amplification products (e.g., polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and probe hybridizations followed by thermal melting analysis) would have difficulty detecting wild-type and mutant alleles. A recently developed PCR technique, allele-specific primer-PCR⁽¹¹⁾, can overcome occasional amplification competition between resistant and susceptible genotypes. In this format, specific primers, each one corresponding to wild type and mutant alleles, are substituted for probes, and each allele is amplified separately.

The prevalence of the primary clarithromycin resistance in Recife was 16.5%, a value close to that found in Southeast Brazil, 16% in São Paulo⁽¹⁵⁾, and 17.3 % in Belo Horizonte⁽¹⁴⁾. The prevalence in Recife increased to 52% in the group of previously treated patients.

These results concerning resistant genotypes, A2143G and A2142G, were unlike those seen in São Paulo⁽¹⁵⁾, but similar to those found in Belo Horizonte⁽¹⁴⁾, where the genotype A2143G also was the most frequent.

In conclusion, MGB TaqMan[®] PCR was a reliable assay to identify clarithromycin-resistant strains of *H. pylori*, even with many susceptible organisms. In Recife, the prevalence of primary clarithromycin resistance in *H. pylori* infections, 16.5%, indicated that pre-treatment susceptibility testing is essential.

Lins AK, Lima RA, Magalhães M. *Helicobacter pylori* resistente à claritromicina no Recife, Brasil, identificada diretamente de biópsias gástricas pela reação em cadeia de polimerase. *Arq Gastroenterol.* 2010;47(4):379-82.

RESUMO – Contexto - Claritromicina é a droga mais eficaz usada na erradicação da infecção pelo *H. pylori*. Devido ao aumento mundial da resistência, o teste de susceptibilidade à claritromicina pré-tratamento é recomendado. **Objetivo** - Determinar a prevalência de *H. pylori* claritromicina resistente em Recife, PE, Brasil. **Método** - De janeiro de 2006 a dezembro de 2007, 114 biópsias gástricas positivas à cultura para *H. pylori* foram diretamente analisadas pela reação em cadeia da polimerase (PCR), para detectar a frequência das mutações em ponto que envolvem a resistência à claritromicina. Os resultados foram comparados com os obtidos pelos E-testes. **Resultado** - Os métodos molecular e fenótipo fenotípico mostraram 111 (97.4%) resultados concordantes, sensíveis ou resistentes. A PCR detectou três (2.6%) espécimes de *H. pylori* com genótipo resistente, diagnosticados erroneamente como sensíveis pelo E-teste. No Recife, baseando-se nos resultados da PCR, a resistência primária à claritromicina foi encontrada em 15 (16.5%) pacientes, esta prevalência também foi observada no sudeste do Brasil. Entre os pacientes previamente tratados, a resistência elevou-se para 52%. A mutação em ponto A2143G foi observada em 20 (71.4%) dos espécimes e a A2142G, em 8 (28.6%). A mutação A2142C não foi encontrada. **Conclusão** - No Recife, a prevalência da resistência a claritromicina, 16.5%, mostrou a necessidade de realização dos testes de susceptibilidade pré-tratamento nas infecções por *H. pylori*.

DESCRITORES - *Helicobacter pylori*. Infecções por helicobacter. Claritromicina. Resistência microbiana a medicamentos. Reação em cadeia da polimerase.

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