ORIGINAL ARTICLE

HELICOBACTER PYLORI GENOTYPING FROM POSITIVE CLOTESTS IN PATIENTS WITH DUODENAL ULCER

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Even though the seroprevalence of *H. pylori* may be high in the normal population, a minority develops peptic ulcer. Colonization of the gastric mucosa by more pathogenic *vac*A strains of *H. pylori* seems to be associated with enhanced gastric inflammation and duodenal ulcer. *H. pylori* genotyping from positive CLOtests was developed to determine the *vac*A genotypes and *cag*A status in 40 duodenal ulcer patients and for routine use. The pathogenic s1b/ m1/ *cag*A genotype was the most frequently occurring strain (17/ 42.5%); only two (5%) patients presented the s2/ m2 genotype, the less virulent strain. Multiple strains were also detected in 17 (42.5%) patients. Multiple strains of *H. pylori* colonizing the human stomach have been underestimated, because genotyping has been performed from cultures of *H. pylori*. We concluded that genotyping of *H. pylori* from a positive CLOtest had the advantages of reducing the number of biopsies taken during endoscopy, eliminating the step of culturing *H. pylori*, and assuring the presence of *H. pylori* in the specimen being processed.

DESCRIPTORS: ***Helicobacter pylori*. Genotype. Duodenal ulcer. CLOtest.**

Helicobacter pylori (*H. pylori*) are curved or spiral-shaped Gram-negative bacteria[1]. The organisms are found close to the mucosal surface, in intercellular positions or caught up in the surface mucus of the stomach[2]. When cultured on solid medium, the bacterium assumes a rod shape; spiral forms may be few or absent. After prolonged culture, coccoid forms predominate[1]. The colonization of the gastric mucosa by *H. pylori* has been associated with chronic gastritis, peptic ulcer disease, and gastric cancer[3-5].

Although the seroprevalence of *H. pylori* may be high in the normal population, a minority develops peptic ulcer[6]. Racial differences in *H. pylori* seroprevalence and peptic ulcer frequency were observed in Singapore; Indians had a higher prevalence of *H. pylori* antibodies but a lower frequency of peptic ulcer than the Chinese. This finding suggested that other environmental or genetic factors may be involved in peptic ulcer disease[7]. Another possible explanation is the fact that some strains are more pathogenic than others[6].

There are two types of *H. pylori*: one (toxin +) can, and the other (toxin -) cannot secrete a vacuolating cytotoxin, which is a protein encoded by the *vac*A (vacuolating cytotoxin) gene[8]. Toxin + strains also produce a 128 kDa protein encoded by the *cag*A gene (cytotoxin-associated gene)[9]. Patients harboring the toxin + strain would be prone to present gastric or duodenal ulceration and gastric cancer[10-13].

Four different families of *vac*A signal sequences (s1a, s1b, s1c, and s2) and two different families of middle-region alleles (m1 and m2) were characterized in different *H. pylori* isolates. The s1c allele was observed exclusively in isolates from East Asia[14]. All possible combinations of these *vac*A regions were identified, with the exception of s2/ m1. Type s1/ m1 strains produced a higher level of cytotoxin activity *in vitro* than the type s1/ m2

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strains; none of s2/ m2 strains produced detectable cytotoxin activity[8,10-12]. However, even though m2 cytotoxin was inactive in the *in vitro* HeLa cell cytotoxicity assay, the m2 cytotoxin was able to induce vacuolization in primary gastric cells[15].

The purpose of our study was to develop a quick technique for routine use of *H. pylori* genotyping from the CLOtest. *H. pylori* genotyping directly from gastric biopsy specimens has previously been described[16-19]. However, the advantages of using the CLOtest for genotyping would be a reduction of the number of biopsies taken during the endoscopy and the assurance of the presence of *H. pylori* in the specimen being processed for genotyping.

PATIENTS AND METHODS

Patients

Forty patients with duodenal ulcer and with positive CLOtests were selected for the study. All patients were positive for *H. pylori*, based on histological findings in gastric biopsies done at the same time that the CLOtest was performed. Twenty patients were male and 20 were female, with a mean age of 48± 13 yr.

CLOtest

The antral mucosal biopsy specimen was inserted into a homemade urease test tube[20]. The urease reagent was prepared by dissolving the following in distilled water to a final volume of 100 mL: 0.010 g yeast extract, 0.0091 g KH_2PO_4, 0.0095 g Na_2HPO_4, 2 g urea, and 15 drops of phenol red 0.5%; the pH of the solution was adjusted to 6.9. The urease reagent was sterilized by filtration, dispensed into 0.5 mL aliquots, and stored at –20°C. If the urease enzyme of *H. pylori* was present in the gastric biopsy, the resulting breakdown of urea caused the pH to rise and the color of the solution to turn from yellow to bright magenta. The urease test tube was examined after a 24 h period.

DNA extraction

After the CLOtest reading at 24 hours, the CLOtest tubes were stored at 4°C until DNA extraction. The whole content of CLOtest including gastric biopsy was collected by centrifugation at 12 000 g for 25 min. The supernatant was discarded, and the pellet was re-suspended in DNA extraction buffer according to Sambrook et al.[21], using the phenol-chloroform method. The DNA pellet was re-suspended in 30 µL TE (10 mmol Tris-HCl pH 8.0, 1 mmol EDTA pH 8.0).

Polymerase Chain Reaction

Genomic DNA (1.2- 6.0 µg) was used as a template in a reaction volume of 50 µL, containing 20 mmol Tris-HCl (pH 8.4), 50 pmol of each primer, 200 µmol of each dNTP, and 2.5 U of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, MD, USA). The Polymerase Chain Reaction (PCR) was performed in a 2400 GeneAmp PCR system (Perkin Elmer, Branchburg, NJ, USA). Amplification was performed under the following conditions for *vac*A (m1, m2, s1a, s1b, s2)[10] and *cag*A[9] (Table 1): initial denaturation at 94°C for 5 min followed by 27 cycles of denaturation at 94°C for 30s, annealing at 53°C for 30s and extension at 72°C for 30s. The final extension at 72°C was for 7 min. Gene Amp® lambda control reagents (Perkin Elmer, Branchburg, NJ, USA); control template lambda DNA and primers were included as positive PCR reaction internal control. Negative PCR reaction internal control was performed by excluding *H. pylori* genomic DNA in one of the PCR reaction tubes. One set of primers (P1 and P2)[16] that amplifies a 26 kDa antigen gene present in all strains of *H. pylori* was used for the negative PCR reaction cases according to the following conditions: initial denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 93°C for 1 min, annealing at 57°C for 2 min and extension at 70°C for 2 min. The final extension at 70°C was for 10 min.

Analysis of PCR products

Five microliters of each PCR mixture were separated by electrophoresis on 2% agarose (GIBCO BRL, Gaithersburg, MD, USA) gels in TAE[21] (0.04 M Tris-acetate, 0.001M EDTA pH 8.0) and 0.5 µg/mL ethidium bromide. TAE was also used for electrophoresis buffer. PCR mixtures and 50 bp DNA ladder (GIBCO BRL, Gaithersburg, MD, USA) were loaded into slots in 6x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 3% glycerol in water)[21].

RESULTS

The PCR technique from the positive CLOtest tubes could be easily applied for the characterization of *H. pylori* strains. *Vac*A and *cag*A genotypes of *H. pylori* were analyzed in 40 duodenal ulcer patients that were diagnosed positive for *H. pylori* by means of histology. Three patients were not included in the study because no PCR product could be obtained, even using one set of primers (P1 and P2) that amplifies a 26kDa antigen present in all strains of *H. pylori* and 6 µg of genomic DNA. The DNA extracted from the biopsies studied, except for the ones that had multiple strains, gave PCR products of expected sizes (Fig. 1).

Analysis of the *vac*A and *cag*A status in these 40 patients (Table 2) revealed that 17 (42.5%) patients had s1b/ m1/ *cag*A, 17 (42.5%) patients had multiple strains, 2 (5%) patients had s1a/ m1/ *cag*A, 2 (5%) patients had s1b/ m2/ *cag*A, and 2 (5%) patients had s2/ m2.

Table 1 - Primers used for genotyping *H. pylori vac*A alleles [10] and *cag*A status [9].

Region	Primer name	Primer sequence	PCR product size
m1	VA3-F	5'- GGTCAAAATGCGGTCATGG-3'	290 bp
	VA3-R	5'- CCATTGGTACCTGTAGAAAC-3'	
m2	VA4-F	5'- GGAGCCCCAGGAAACATTG-3'	352 bp
	VA4-R	5'-CATAACTAGCGCCTTGCAC-3'	
s1a	SS1-F	5'- GTCAGCATCACACCGCAAC-3'	190 bp
	VA1-R	5'- CTGCTTGAATGCGCCAAAC-3'	
s1b	SS3-F	5'- AGCGCCATACCGCAAGAG-3'	187 bp
	VA1-R	5'- CTGCTTGAATGCGCCAAAC-3'	
s2	SS2-F	5'- GCTAACACGCCAAATGATCC-3'	199bp
	VA1-R	5'- CTGCTTGAATGCGCCAAAC-3'	
*Cag*A	CAG-1	5'- AGACAACTTGAGCGAGAAAG-3'	320bp
	CAG-2	5'- TATTGGGATTCTTGGAGGCG-3'	
Ag	P1	5'-TGGCGTGTCTATTGACAGCGAGC-3'	298bp
	P2	5'- CCTGCTGGGCATACTTCACCATG-3'	

Ag: DNA sequence of a species-specific protein antigen of 26kDa molecular weight that was present in all strains of *H. pylori*[16].

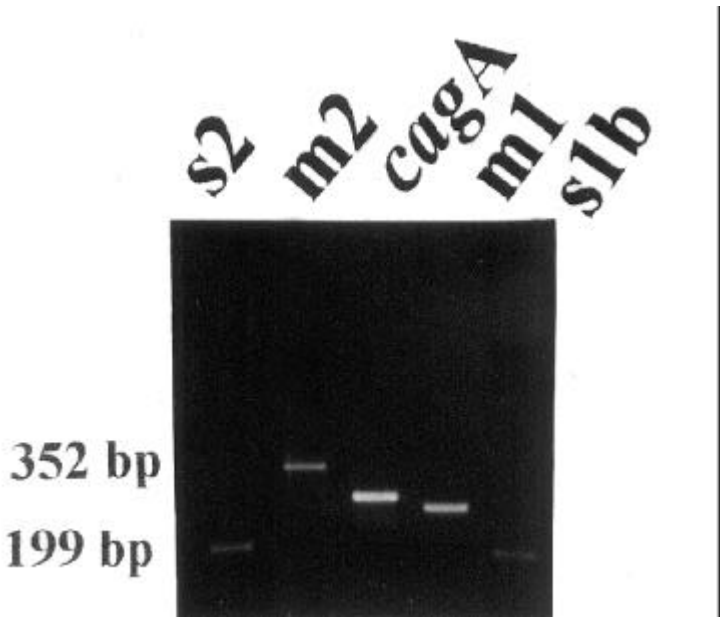

Figure 1 - PCR genotyping of *vac*A and *cag*A status from one case with the s2/ m2 strain and another case with the s1b/ m1/ *cag*A strain. Primers described in Table 1 were used for PCR reaction.

DISCUSSION

In the present study, we described the analysis of *H. pylori* strains by PCR from positive homemade CLOtests in 40 duodenal ulcer patients. Compared to using gastric biopsies, the possibility of using the CLOtest for PCR would have the following advantages: 1) the reduction of the number of biopsies taken during endoscopy; 2) the assurance of the presence of *H. pylori* in the specimen being processed, and 3) the avoidance of time-consuming culturing of the strains.

Table 2 - *vac*A and *cag*A status of *H. pylori* strains from CLOtests of 40 patients with duodenal ulcer.

Genotype status	Patients (%)
s1b/ m1 / *cag*A	17 (42.5%)
s1a/ m1 / *cag*A	2 (5%)
s1b/ m2 / *cag*A	2 (5%)
s2/ m2	2 (5%)
Multiple strains	17 (42.5%)

The determination of the *vac*A genotype was possible in more than 90% of the positive CLOtests, in agreement with another report[18], since only 3 patients were not included in this study because no PCR product could be obtained. A low density of *H. pylori* on the gastric mucosa could be responsible for a negative PCR, as the sensitivity of the PCR detection of *H. pylori* in gastric biopsies was approximately of 70-100 bacterial cells[16,18]. One set of primers (P1 and P2)[16] that amplifies a 26 kDa antigen present in all strains of *H. pylori* was also used to make sure that the negative PCR reactions were due to a low density of *H. pylori*, instead of the genotyping technique itself. Actually, typing of the *vac*A gene was not possible in five strains by other authors[22], because of a 61-bp insertion in the signal region of two strains, and for unknown reasons in the others.

The s1b/ m1/ *cag*A genotype was the most frequent strain (42.5%) observed in these patients; a similar result has already been described in another study of *H. pylori* strains from Brazil[23]. A high prevalence of *vac*A s1b alleles was also reported in South Africa[13] and Portugal[24]. However, *vac*A

s1a alleles were more prevalent in Japan[22] and in Northern and Eastern Europe[23].

H. pylori strains of *vac*A signal sequence type s1a are associated with more gastric inflammation and duodenal ulceration than are the s1b type. *Vac*A s2 strains are associated with less inflammation and lower ulcer prevalence[6]; in the present study, a low prevalence was also observed, as only two (5%) patients with duodenal ulcer had *vac*A s2/ m2 strain.

Multiple strains were detected at the same rate (42.5%) as the s1b/ m1/ *cag*A genotype; the clinical relevance of multiple strains in gastric biopsies should be evaluated, because the virulence-associated genotypes of the strains was correlated with the clinical outcome of the gastrointestinal disease in some studies[10-13], but not in others[5, 14, 22, 25].

The detection of a high frequency of multiple strains could be explained by the fact that genotyping was performed directly from gastric biopsies; other authors[12,16-19] also obtained more than one strain when using the same approach. In contrast, when the step of *H. pylori* culturing preceded genotyping, a single strain may have been picked up; thus, the frequency of multiple strains in the stomach might have been underestimated. According to Van Doorn et al[23] and Figura et al[26], purification of *H. pylori* strains by culturing from a single colony universally results in the detection of a single *vac*A genotype; however, when the strains used are not purified from a single colony, they may reflect the presence of multiple strains in the host's stomach.

Another aspect that has to be considered in the disease outcome (duodenal or gastric ulcer, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphoma) of positive *H. pylori* patients is the individual host's response to the *H. pylori* infection. The cellular and humoral immune responses that are mounted against *H. pylori* are vigorous; polymorphonuclear leukocytes and macrophages, as well as T and B lymphocytes, infiltrate the gastric mucosa, and have been shown to modify gastric acid secretion[27]. Different gastric acid responses to *H. pylori* have been associated with variations in the gastritis patterns that seem to determine disease outcome[28,29]. Thus, the immune response of the host does not clear the infection and leaves the host prone to complications resulting from chronic inflammation[30-33].

In a mechanism known as antigenic mimicry, highly conserved immunogenic molecules expressed by infectious pathogens may act as a trigger for the induction of humoral and cellular immune responses that cross-react with host cellular antigens. *H. pylori* seems to be very effective in inducing antigenic mimicry; antibodies against *H. pylori* have been found to cross-react with both antral mucosal cells and gastrin-producing cells. Such autoantibodies were detected both in human and in experimental work in rodents[34].

In conclusion, genotyping from a homemade CLOtest was successfully developed for routine use in our laboratory. Even though more virulent strains of *H. pylori* were found in duodenal ulcer patients, the host immune responses to *H. pylori* should be further evaluated.

RESUMO RHCFAP/3018

MATTAR R e col. – A genotipagem do *H. pylori* de testes CLO positivos em pacientes com úlcera duodenal. **Rev. Hosp. Clín. Fac. Med. S. Paulo 55**(5):155-160, 2000.

Apesar da prevalência do *H. pylori* na população normal ser alta, somente uma minoria desenvolve úlcera péptica. A colonização da mucosa gástrica por cepas mais patogênicas de *H. pylori* tem sido associada com maior inflamação gástrica e úlcera duodenal. A genotipagem do *H. pylori* de testes CLO positivos foi estabelecida para se determinar os genótipos *vac*A e *cag*A em 40 pacientes com úlcera duodenal e para uso na rotina. O genótipo patogênico s1b/m1/*cag*A foi o mais freqüente (17/ 42,5%); apenas dois (5%) pacientes apresentaram o genótipo s2/m2, o que é o menos virulento. Cepas múltiplas também foram detectadas em 17 (42,5%) pacientes. Cepas múltiplas colonizando o estômago têm sido subestimadas, pelo fato das genotipagens serem geralmente realizadas a partir de culturas de *H. pylori*. Nós concluímos que a genotipagem do *H. pylori* a partir de testes CLO positivos tem as vantagens de reduzir o número de biópsias durante a endoscopia, eliminar a etapa de cultura do *H. pylori*, e a certeza da presença do *H. pylori* na amostra que está sendo processada para a genotipagem.

DESCRITORES: ***Helicobacter pylori*. Genótipo. Úlcera duodenal. Testes CLO.**

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