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COMPARISON OF HISTOLOGICAL AND MOLECULAR DIAGNOSIS OF *HELICOBACTER PYLORI* IN BENIGN LESIONS AND GASTRIC ADENOCARCINOMA

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ABSTRACT

Helicobacter pylori colonization is associated with chronic gastritis, peptic ulcers, intestinal metaplasia, adenocarcinoma and lymphoma of the stomach. The objective of this study was to compare the results of the routinely used histology with molecular diagnosis for the detection of *H. pylori*. Eighty samples from gastric lesions (chronic gastritis, atrophic gastritis, gastric ulcer, and intestinal metaplasia), 18 gastric adenocarcinoma and 10 normal mucosa *H. pylori*-negative (control) samples were obtained. All samples were examined histologically (hematoxylin-eosin and Giemsa staining), and PCR amplifications of the species-specific antigen gene (H3H4) and urease A gene segment (H5H6) of *H. pylori* were made, using the human gene *CYP1A1* for DNA quality control. In the benign lesion and adenocarcinoma the infection was detected in 43% (42/98) and 71% (70/98) by histological and molecular diagnosis (p=0.0001), respectively. The PCR test detected *H. pylori* in 27.5% (22/80) of the benign gastric lesions and in 50% (9/18) of the gastric adenocarcinoma cases, the histological diagnosis being negative for this bacterium. About 2.5% of the samples, exclusively from benign lesions and with a positive histological diagnosis, showed negative molecular results for both primers. Statistically significant differences were found between the histological and the molecular method in intestinal metaplasia (p=0.0461) and gastric adenocarcinoma (p=0.0011), due to underdetection of *H. pylori* by the histological method, which is probably due to the low density of the bacterium as a consequence of the severe atrophy of the gastric mucosa. Our findings suggest that PCR is the more efficient method for the assessment of *H. pylori* infection, especially in metaplasia and gastric adenocarcinoma.

Key words: *Helicobacter pylori*, gastric lesions, adenocarcinoma, histological diagnosis, PCR

INTRODUCTION

Helicobacter pylori is a Gram-negative spiral flagellated bacterium that colonizes the gastric mucosa. It induces chronic gastritis and is associated with the development of gastric and duodenal ulcers, gastric carcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (11,38). This bacterium is the main etiologic agent in 95% of chronic gastritis cases and in 20% of gastric ulcers, and has been responsible for an approximately ninefold increase of the risk for gastric cancer (21,31). Although 60-80% of gastric cancer cases have a positive diagnosis for *H. pylori* (21), the infection is not the sole triggering factor, since the risk is only about 1-2% in infected people (31). Thus, other factors should be considered, such as the age of onset of the infection, host factors, and the genotype of *H. pylori* strains.

Epidemiologic studies showed that *H. ylori* colonizes over 50% of the world population, affecting 40-50% of the population in the industrialized countries and 70-90% in developing countries (36).

Transmission occurs via person-to-person passage, usually during childhood. Factors influencing the transmission of *H.*

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pylori include socioeconomic status, density of living, educational level, sanitation and genetic predisposition (3).

The association between *H. pylori* infection and increased risk for gastric adenocarcinoma is not well understood. This bacterium produces large amounts of the enzyme urease that converts urea into carbon dioxide and ammonia, neutralizing the gastric acid and allowing the bacterium to colonize the stomach (24). *H. pylori* also stimulates the production of nitric oxide (NO) by macrophages and neutrophils. NO is a potent endogenous mutagen, because it causes genetic damage, whereas ammonia promotes gastric cell proliferation (34).

Other mechanisms also appear to be related with *H. pylori* colonization of the stomach and risk for developing gastric cancer, such as the production of anti-gastric auto-antibodies, correlated with the severity of gastritis and gastric mucosal atrophy (14), decreasing the intragastric levels of the antioxidant ascorbic acid which scavenges free oxygen metabolites and also inhibits the conversion of nitrite to N-nitrosamines (20) and infection by vacA- or CagA-producing *H. pylori* strains.

Many invasive and non-invasive methods are available for the detection of *H. pylori* infection. Invasive methods require endoscopy to obtain biopsies of gastric tissues, in which *H. pylori* can be diagnosed by urease activity, histology, PCR test or culture (19,37). Noninvasive techniques to detect the bacterial infection include carbon urea breath tests (CUBT), antigen stool tests and serological methods for the detection of anti-*H. pylori* antibodies (35).

The aim of this study was to compare the results of histology (Giemsa staining) used as a routine to detect *H. pylori* with those of molecular diagnosis, obtained by polymerase chain reaction (PCR) using two sets of *H. pylori* oligonucleotides in benign gastric lesions and gastric adenocarcinoma.

MATERIALS AND METHODS

Samples

One hundred and eight samples (10 from normal mucosa, 31 from non-atrophic gastritis, 13 from atrophic chronic gastritis, 18 from gastric ulcers, 18 from intestinal metaplasia, and 18 from gastric adenocarcinoma), obtained by biopsy or surgery fragments of fresh or paraffin-embedded material (seven cases) were evaluated. Samples of gastric lesions (92.5% from antrum and 7.5% from body) and adenocarcinoma (72% distal and 28% proximal) were collected from 54 men and 44 women (range 2-94 years, mean age 55.8 years). Histologically normal and *H. pylori*-negative mucosa biopsies were obtained from 10 individuals with a mean age of 43 years (ranging from 19 to 75 years). In most cases, the fragments were obtained from the antrum (74.4% cases) of non-necrotic area. All samples were collected at the Hospital de Base of São José do Rio Preto, SP, Brazil. The endoscopic forceps were sterilized between experiments in 2% glutaraldehyde solution for a minimum of 20 minutes. This study was approved by the National Research Ethics Committee, and written informed consent was obtained from all patients.

Histology

Hematoxylin-eosin (H&E) staining was used for the diagnosis and classification of gastritis (9) and adenocarcinoma, according to Lauren (22). A modified Giemsa staining was used to visualize *H. pylori*.

DNA extraction and PCR for diagnosis of *H. pylori*

DNA was extracted by the phenol-chloroform method, after digestion with proteinase K (29). PCR assays were performed separately, with approximately 100ng of total DNA, using three different sets of oligonucleotides. One of them amplifies a 312bp segment of the gene *CYP1A1* (1) as a quality control DNA, the other (H3H4) amplifies a 298bp product of the gene encoding species-specific *H. pylori* antigen (18), and the last one (H5H6) amplifies a 411bp fragment corresponding to the urease A gene (6). Positive and negative controls were used in all experiments. The PCR products were separated by 7.5% polyacrylamide-gel electrophoresis, followed by silver nitrate staining. The assay was considered positive when at least one of the bacterial PCR products was present (12).

Statistical analysis

The Fisher exact test was used to determine statistical significance. Values of p lower than 0.05 were considered significant.

RESULTS

Table 1 shows the results of histology and molecular diagnosis of *H. pylori* infection in the samples from benign lesions and gastric adenocarcinoma, except the normal mucosa sample that was *H. pylori*-negative for both methods.

In the benign lesions and adenocarcinoma samples the infection was present in 43% (42/98) and 71% (70/98), respectively, according to histological and molecular diagnosis (p=0.0001). The PCR products were concordant for both primers in 54% (53/98) of cases. The infection was considered positive when at least one of the bacterial PCR products was amplified.

The PCR test detected *H. pylori* in 27.5% (22/80) of the benign gastric lesions and in 50% (9/18) of the gastric adenocarcinoma samples, with negative histological diagnosis for this bacterium. About 2.5% of the samples, exclusively from benign lesions and with a positive histological diagnosis, showed negative molecular results for both primers. Statistically significant differences were found between the histological and the molecular method in intestinal metaplasia (p=0.0461), gastric adenocarcinoma (p=0.0011), and all samples jointly (p=0.0001), but not with regard to non-atrophic (p=0.0634) and atrophic (p=0.6559) gastritis and gastric ulcer (p=0.0606).

Table 1. A comparison of relative and percentage frequencies and Fisher exact test values of *H. pylori* infection, as diagnosed by histology and molecular diagnosis in benign lesions and gastric adenocarcinoma.

Samples	N	Histology (%)		Molecular (%)						
				H3H4		H5H6		H3H4/H5H6		*p*
		HP-	HP+	HP-	HP+	HP-	HP+	HP-	HP+	
Non-atrophic gastritis	31	19/31 (61.2%)	12/31 (38.7%)	13/31 (41.9%)	18/31 (58.0%)	15/31 (48.3%)	16/31 (51.6%)	12/31 (38.7%)	19/31 (61.3%)	0.0634
Atrophic gastritis	13	5/13 (38.5%)	8/13 (61.5%)	4/13 (30.7%)	9/13 (69.2%)	6/13 (46.1%)	7/13 (53.8%)	5/13 (38.5%)	8/13 (61.5%)	0.6559
Gastric ulcer	18	7/18 (38.9%)	11/18 (61.1%)	3/18 (16.6%)	15/18 (83.3%)	3/18 (16.7%)	15/18 (83.3%)	2/18 (11.1%)	16/18 (88.9%)	0.0606
Intestinal metaplasia	18	11/18 (61.1%)	7/18 (38.9%)	6/18 (33.3%)	12/18 (66.7%)	5/18 (27.7%)	13/18 (72.2%)	5/18 (27.8%)	13/18 (72.2%)	0.0461
Adenocarcinoma	18	14/18 (77.8%)	4/18 (22.2%)	9/18 (50.0%)	9/18 (50.0%)	9/18 (50.0%)	9/18 (50.0%)	4/18 (22.2%)	14/18 (77.8%)	0.0011
TOTAL	98	56/98 (57.1%)	42/98 (42.8%)	35/98 (35.7%)	63/98 (64.3%)	38/98) (38.7%)	60/98 (61.2%)	28/98 (28.6%)	70/98 (71.4%)	0.0001

HP- = *H. pylori*-negative; HP+ = *H. pylori*-positive; N = number of samples; H3H4/H5H6= HP+ in at least one PCR.

DISCUSSION

Several mechanisms were postulated to link *H. pylori* infection and gastric carcinogenesis, such as increased ammonia production leading to gastric mucosal atrophy (7), decreased intragastric levels of the antioxidant ascorbic acid (3), increased production of mutagenic free radicals (8), and increased cellular proliferation and occurrence of apoptosis in gastric epithelial cells (17).

H. pylori may be present in the initial steps of gastric tumorigenesis, and this infection may precede mutations or deletions of genes engaged in this process, such as *TP53, c-ERBB-2, CDH-1* and *cyclin D1* (40).

At the moment, no single test can be absolutely relied upon to detect colonization by *H. pylori*, and a combination of two tests is recommended if feasible (28). Many invasive and non-invasive methods were developed. In clinical practice, histology and urea breath test are the most used ones (26). The sensitivity of invasive methods depends on some factors such as the number of biopsies collected, density of bacterium in the biopsy specimens, persistence of *H. pylori* in the endoscopic equipment and presence of microorganisms other than *H. pylori* (28).

The rapid urease test, histology and culture may give false-negative results, due to low density of *H. pylori* present in the specimens. False-negative results of the rapid urease test may also be observed when *H. pylori* is present in the coccoid form, thus lacking urease activity. On the other hand, the presence of other microorganisms with urease activity, such as *Proteus*, *Yersinia*, *Klebsiella* and *Pseudomonas*, may be held responsible for a false-positive urease test, while spiral Gram-negative bacteria, such as *Campylobacter jejuni*, that may resemble *H. pylori*, can lead to false-positive histology (28).

The PCR assay has so far not been widely used in clinical practice, despite its high specificity and sensitivity in detecting genetic information of *H. pylori* (15,30). The presence of genes encoding proteins that enhance the virulence of the strain (5,39) or mutations that confer antibiotic resistance will also be useful in the choice of the appropriate therapy.

Our study detected 43% *H. pylori*-positive samples by histological assay. About 31.6% (31/98) of the cases which tested negative for *H. pylori* by Giemsa staining were found to be positive by PCR. This could be explained by the fact that *H. pylori* colonizes the gastric mucosa in a patchy manner, or that the number of present bacteria was very small or, less likely, by contamination of the biopsies by *H. pylori* DNA from the endoscopes (33). This last hypothesis is unlikely, since Paoluzzi *et al.* (28) did not detect *H. pylori* DNA in aliquots of the water used in maneuvers for cleaning endoscopic equipment, after disinfection with 2% glutaraldehyde. In the present study, the same cleansing routine was thoroughly employed.

As mentioned earlier, PCR detection of bacterial DNA is not yet widely used in clinical diagnosis, but is has been proven

reliable for the detection of *H. pylori* DNA in gastric biopsies (13), gastric juice (10), and feces (2).

Nogueira *et al.* (27) studies mucosal fragments of forty patients receiving gastrectomy for gastric carcinoma. Nineteen cases were studies by microbiology method and *H. pylori* was detected in 82.5% of the cases. These authors examined areas without atrophy or with minor atrophic changes. In our study, the lower percentage of *H. pylori*-positive samples by histological assay might be explain for collection of samples regardless presence of atrophy, sensitivity of method and the variation in *H. pylori* infection rates.

In the present study, the frequency of *H. pylori*-positive samples detected by PCR was 71% (70/98). Other studies carried out in the Brazilian population have reported differences in the frequency of *H. pylori* infection, probably due to different factors showing regional variation, such as socioeconomic status, educational level, sanitation and also the methods diagnosed employed. A percentage of 86.6% was found in Campinas-SP (23), of 76.3% in São Paulo-SP (16), 59.5% in Rio de Janeiro-RJ (32), 83% in Santa Maria-RS (25), and 96% in São Luis-MA (4).

Ours findings suggest that the PCR method is more efficient to assess *H. pylori* infection than histology, especially in metaplasia and gastric adenocarcinoma. The differences between these two methods may be explained by changes in the gastric mucosa, leading to a decrease in the secretion of normal mucus, thus making it difficult for *H. pylori* to survive in this altered environment. The bacteria then migrate to another site of the stomach or the infection comes to an end. In metaplastic regions, a minor affinity with the acid mucus is responsible for insufficient presence of *H. pylori.*

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RESUMO

Comparação dos diagnósticos histológico e molecular do *Helicobacter pylori* em lesões benignas e adenocarcinomas gástricos

A colonização do *Helicobacter pylori* está associada com gastrite crônica, úlcera péptica, metaplasia intestinal, adenocarcinoma e linfoma gástrico. O objetivo desse estudo foi comparar os resultados do diagnóstico histológico usado de rotina na detecção do *H. pylori* com o diagnóstico molecular. Foram utilizadas amostras de 80 lesões gástricas (gastrite crônica, gastrite atrófica, úlcera gástrica e metaplasia intestinal), 18 amostras de adenocarcinoma gástrico e 10 amostras de mucosa gástrica normal *H. pylori* negativas (controle). Todas as amostras foram avaliadas histologicamente (coloração com hematoxilina-eosina e Giemsa) e pela PCR com a amplificação dos genes antígeno espécie-específico (H3H4) e urease A (H5H6) do *H. pylori* e pelo gene humano *CYP1A1*, como controle da qualidade do DNA. Nas amostras de lesão benigna e adenocarcinoma gástrico, a infecção foi detectada em 43% (42/98) e 71% (70/98), respectivamente, para os diagnósticos histológico e molecular (p=0,0001). O teste de PCR detectou o *H. pylori* em 27,5% (22/80) das lesões gástricas benignas e em 50% (9/18) dos adenocarcinomas gástricos, com diagnóstico histológico negativo para essa bactéria. Cerca de 2,5% das amostras, exclusivamente de lesões benignas, com diagnóstico histológico positivo apresentaram resultado molecular negativo, para ambos os primers. Diferenças estatisticamente significantes foram encontradas entre os métodos histológico e molecular, em metaplasia intestinal (p=0,0461) e adenocarcinoma gástrico (p=0,0011), devido à subdetecção do *H. pylori* pelo método histológico, e provavelmente pela baixa densidade da bactéria conseqüente à atrofia severa da mucosa gástrica, nesses dois tipos de lesões. Nossos achados demonstram que o método de PCR é mais eficaz para diagnosticar a infecção por *H. pylori*, principalmente, em metaplasia intestinal e adenocarcinoma gástrico.

Palavras-chave: *Helicobacter pylori*, lesões gástricas, adenocarcinoma, diagnóstico histológico, PCR

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