Short Communication

Quantitative real-time PCR for the clinical detection of *Helicobacter pylori*

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Abstract

Accurate diagnosis of *Helicobacter pylori* infection is very important in both clinical practice and research. We evaluated the sensitivity of real-time PCR (RT-PCR) for the detection and quantification of *Helicobacter pylori* using DNA from 91 human gastric biopsy samples divided into three groups: 46 biopsies from untreated patients who according to the references methods were considered *H. pylori*-negative (group A); 35 biopsies from patients previously treated against *H. pylori* and considered to be cured by "gold standard" tests (group B); and 10 biopsies from patients *H. pylori*-positive by all available methods (group C). The sensitivity of the RT-PCR assay was higher than that of standard methods. Of the 81 patients considered to be uninfected according to the references methods, 16 were *H. pylori*-positive by PCR, 10 of which were patients who had received *H. pylori* eradication therapy and 6 were untreated patients. Based on these findings we recommend that RT-PCR should be use in addition to standard methods in clinical studies to monitor the results of *H. pylori* eradication therapy.

One of the most common chronic infections in humans is the bacteria *Helicobacter pylori* (Graham, 2000), which is considered to be the major causative agent of gastritis and a key etiological factor in peptic ulcer disease, gastric cancer and MALT (Mucosa-Associated Lymphoid Tissue) [Undefined acronym.] lymphoma (Blaser, 1990; Marshall, 1994). Infection with *H. pylori* can be effectively treated by the combination of a proton pump inhibitor with multiple antibiotics but although the bacteria can be eradicated in up to 90% of patients side effects, poor compliance and resistance to the antibiotics used are common causes of treatment failure (Megraud and Doermann, 1998; de Boer and Borody, 2000).

Accurate diagnosis of *H. pylori* infection is very important in both clinical practice and for research purposes and several invasive and noninvasive methods for *H. pylori* detection are currently available. In all invasive procedures gastric biopsies are required coupled with tests such as the rapid urease test (RUT), bacteriological culture, tissue histology and PCR-based diagnostic, while the noninvasive tests include direct fecal antigen detection, serology, urea breath testing and PCR of saliva, dental plaques or stool samples (Versalovic, 2003). Although the sensitivity and specificity of diagnostic tests for *H. pylori* detection vary from satisfactory to very good, several studies have shown that the sensitivity of methods such as bacterial culture, RUT, histology and stool antigen tests could be unreliable when few bacteria are present (Ho *et al.*, 1991). Additionally, serology cannot detect the clearance of *H. pylori* and urease assays can lead to non-specific results due to the presence of other urease-positive bacteria and false negative results have also been reported in individuals taking proton pump inhibitors (Mobley *et al.*, 1991; Graham *et al.*, 2003). Methods based on molecular biology are considered highly specific and sensitive tests, and many PCR-based assays have been developed to detect *H. pylori* DNA in gastric biopsies, saliva and stool samples (Li *et al.*, 1996; Pacheco *et al.*, 2001). However, this technique is able to detect specific fragments but not viable bacteria and its sensitivity also depends on the amount of bacterial DNA (Pacheco *et al.*, 2001).

Recently, real-time PCR (RT-PCR) quantitative assays have been developed to quantify *H. pylori* in gastric and stool samples (He *et al.*, 2002; Kobayashi *et al.*, 2002; Lascols *et al.*, 2003; Mikula *et al.*, 2003; Schabereiter-Gurtner *et al.*, 2004). These methods allow rapid and accurate quantification of *H. pylori* and have increased in importance because they can facilitate the monitoring of

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therapy and even measure the effectiveness of vaccines against *H. pylori* (He *et al.*, 2002).

The sensitivity and specificity of the currently available tests for *H. pylori* detection may be affected by several factors and more reliable methods are desirable. We described the applicability and sensitivity of RT-PCR for *H. pylori* detection and quantification in 81 biopsies from patients considered to be non-infected and 10 biopsies from *H. pylori*-positive patients.

We collected 91 human gastric biopsy samples from patients with dyspeptic symptoms who presented themselves at the Clinical Pharmacology and Gastroenterology Unit of São Francisco Medical School (Bragança Paulista, SP, Brazil). This study was approved by the Ethics Committee of São Francisco University, and was done in accordance with the Declaration of Helsinki. Each patient signed a written informed consent prior to entering the study.

All data regarding *H. pylori* detection (RUT, histology and culture) were available and have been published previously (Mendonca *et al.*, 2000; Ecclisatto *et al.*, 2002). We determined *Helicobacter pylori* infection using rapid urease test in one antral biopsy (Probac, São Paulo, Brazil), histological evaluation (hematoxylin and eosin and Giemsa staining) and culture using two samples (one for each method) from the antrum, corpus and fundus.

The 91 human gastric biopsy samples (81 uninfected, 10 infected) formed three groups: 46 biopsies from untreated patients who according to the references methods were *H. pylori*-negative (group A); 35 biopsies from patients previously treated against *H. pylori* and considered to be cured by "gold standard" tests (group B); and 10 biopsies from patients *H. pylori*-positive by all available methods (group C). The patients from group B were treated twice a day for 7 days with lansoprazole 30 mg (Ogastro®, Abbott, Brazil), amoxicillin 1000 mg (Amoxil®, Smith Kline Beecham, Brazil) and clarithromycin 500 mg (Klaricid®, Abbott, Brazil). The patients were re-evaluated at the end of therapy by a second endoscopic examination 30 days following successful completion of the therapy in order to assess *H. pylori* eradication and ulcer healing. Healing of peptic lesions was considered as the absence of ulcers in previously injured mucosa, or the presence of a definite scar. Antrum, corpus and fundus biopsies were taken to determine the presence of *H. pylori* as previously described. Eradication was defined by a negative culture, histology and urease test.

Frozen biopsy samples were thawed at room temperature transferred to a microcentrifuge tubes and centrifuged at 10.000 g for 5 min. The supernatant was decanted and 300 µL of extraction buffer (containing 20 mM Tris-Hcl (pH 8.0) and 0.5% (v/v)Tween 20) was added and the pellet resuspended by vortexing, after which Proteinase K (Sigma Chemical CO., St. Louis, MO) was added to a final concentration of 0.5 mg mL^{-1}. After overnight incubation at 37 °C the DNA was extracted by a phenol-chloroform method and the absorbance at 260/280 nm determined for each sample.

The DNA samples obtained were subjected to a real-time PCR, performed in an iCycler IQ (Bio-Rad, Hercules, CA, USA), data acquisition and analysis were carried out using the Real-Time Detection System Software (Bio-Rad). The RT-PCR was targeted at the 26 KDa *Helicobacter* species-specific antigen (*SSA*) gene (O'Toole *et al.*, 1991) and a double-stranded DNA-specific dye SYBR Green I was used. The 50 µL reaction mixture was composed of follows: 25 µL of iQ SYBR® Green Supermix (Bio-Rad), 50 nM of each primer (forward: 5'-TGGCG TGTCTATTGACAGCGAGC-3', reverse: 5'-CCTGCTG GGCATACTTCACCATG-3') previously described (Mikula *et al.*, 2003), 1 µL of extracted DNA (200 ng).The reaction was cycled with preliminary denaturation for 5 min at 95 °C, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 65 °C for 30 s and primer extension at 72 °C for 30 s. This was followed by melting point analysis of the double-stranded amplicons consisting of 40 cycles of 1 °C decrement (45 s each) beginning at 95 °C. Positive controls consisted of genomic DNA extracted from *H. pylori* reference strains 26695 and J99, kindly provided by dr. Johannes G. Kusters (Erasmus Medical Center, Rotterdam, The Netherlands) and negative controls were provided by DNA isolated from blood samples and gastric mucosa of uninfected mice (strain C57BL/6,CEMIB/ UNICAMP, Campinas, SP, Brazil).

A significant change in fluorescence accompanies the melting curve of the double-stranded PCR products and the rate of change (d) of fluorescence (F) in the reaction with time (T) is given by the first derivative dF/dT, a plot of -dF/dT against temperature displaying these changes as distinct peaks.

To evaluate the detection limit of this reaction, serially diluted samples of *H. pylori* DNA ranging from 10^{-1} to 10^6 fg were used as a template. The efficiency was determined by standard curves on the basis of 10-fold serial dilutions of *H. pylori* DNA in a background of 200 ng of human DNA. Each sample was tested in triplicate and the mean calculated to obtain the final threshold cycle (Ct), *i.e.*, the point at which sample fluorescence rises above the background level. A standard curve, constructed by plotting the log of the initial DNA concentration versus the Ct value was used to determine *H. pylori* DNA concentrations. Comparisons of Ct values and the number of bacterial cells between the three groups were performed with the *t* test, a p value of < 0.05 being considered statistically significant.

The sensitivity of the RT-PCR assay was determined by adding serial 10-fold dilutions ranging from 10^{-1} to 10^6 fg of *H. pylori* chromosome DNA to a background of human DNA. The lowest *H. pylori* DNA concentration detected was 10 fg, equivalent to 5 bacterial cells (Mikula *et al.*, 2003; Schabereiter-Gurtner *et al.*, 2004). In a range between 10 to 10^6 fg correlation coefficients, obtained by lin-

ear regression analysis from all independent experiments, were higher than $R^2 = 0.99$, indicating highly efficient and reproducible reactions. The specificity of the reaction was determined by melting curve analysis and in all dilutions a single product was observed.

The number of bacterial cells and amount of DNA present in each sample were quantified by interpolation of the corresponding Ct values in the standard curves. The RT-PCR results from *SSA* amplification showed that of the 81 patients considered to be uninfected according to the references methods 16 were *H. pylori*-positive by RT-PCR, of which 6 (13%) were group A untreated patients considered to be *H. pylori* negative by the reference methods and 10 (29%) were group B treated patients also considered to be *H. pylori* negative by standard methods. As expected, all group C patients were *H. pylori* positive by RT-PCR.

The number of bacterial cells per 200 ng of total DNA were quantified by real-time PCR and varied from 49 to 160 among the group B patients (the lowest *H. pylori* infection rates) to between 150 and 1400 in the group A patients and from 1000 to 31000 in the *H. pylori*-positive group C patients with the highest infection rates (Figure 1). Additionally, statistical analysis showed that the Ct and the bacterial load were significantly different among the patients from groups A, B and C ($p < 0.02$). The reproducibility of the RT-PCR assay was also evaluated among the 26 *H. pylori*-positive patients (*i.e.* the 16 detected by RT-PCR plus the 10 in group C) with analysis of variance showing that there was no significant difference within or between runs, thus indicating that the assays were reproducible (data not shown).

Recently, five studies have shown that RT-PCR can be used to detect and quantify *H. pylori* DNA directly from gastric biopsy samples (He *et al.*, 2002; Kobayashi *et al.*, 2002; Lascols *et al.*, 2003; Mikula *et al.*, 2003; Schabereiter-Gurtner *et al.*, 2004). Additionally, RT-PCR has been validated for the quantitative analysis of the low level of bacterial infection in immunized and experimentally infected mice, suggesting that this methodology may also be
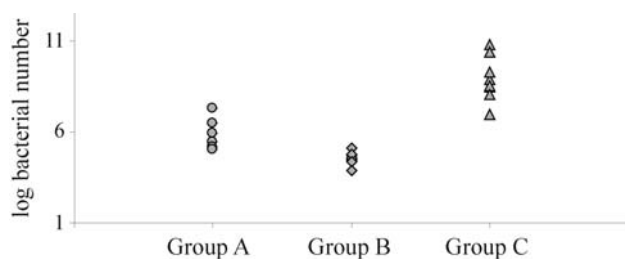

Figure 1 - Number of *Helicobacter pylori* per 200 ng of total DNA for the different biopsy sample groups. Group A, consisting of 46 biopsies from untreated patients who according to the references methods were considered *H. pylori*-negative, showed an intermediary level of infection. Group B, made up of 35 biopsies from patients previously treated against *H. pylori* and considered to be cured by "gold standard" tests, demonstrated a low level of infection. Group C, containing 10 biopsies from patients regarded as *H. pylori*-positive by means of all used methods, exhibited a high level of infection.

useful for the evaluation of new anti-*H. pylori* drugs and vaccines (Mikula *et al.*, 2003).

Our results show that the sensitivity of the RT-PCR assay was higher than that of the standard reference methods RUT, bacteriological analysis and histology, supporting the findings of other workers (He *et al.*, 2002; Lascols *et al.*, 2003; Schabereiter-Gurtner *et al.*, 2004). Indeed, 16 of the 81 "uninfected" patients actually gave large numbers of *H. pylori* DNA copies when assessed by RT-PCR.

Standard methods are also useful for quantification, although each technique has its drawbacks. Bacteriological culture and histology allow only the semi-quantitative determination of infection, and both require experienced professionals for their execution and the interpretation of results (Christensen *et al.*, 1992; Atherton *et al.*, 1996; El-Zimaity *et al.*, 1996; Leodolter *et al.*, 2001). On the other hand, the RT-PCR assay can accurately detect and quantify five bacterial cells per 200 ng of human DNA as previously described (Mikula *et al.*, 2003; Schabereiter-Gurtner *et al.*, 2004). Our results indicated that there was a significant difference in the bacterial density between the three groups analyzed. As expected, patients belonging to group C had the highest *H. pylori* population. The differences detected among the supposedly eradicated group (B) and untreated patients (A) may be attributed to the drugs used for eradication (He *et al.*, 2002) and/or sampling errors that might generate differences in colonization density (Nedenskov-Sorensen *et al.*, 1991). An additional possibility is that some group A patients took, but did not report, proton pump inhibitors, leading to false negative results in the currently used tests for *H. pylori* (Graham *et al.*, 2003).

Although it has been shown that the detection sensitivity of "gold standard" methods for *H. pylori* is highly reduced when the density of *H. pylori* is low and their distribution is "patchy" (Leodolter *et al.*, 2001; Vaira *et al.*, 2002) our results indicated that biopsies from all the patients considered to be negative had an *H. pylori* density higher than 10000 cells, as previously reported by He *et al.* (2002). Growing evidence supports the concept that *H. pylori* exists in two viable forms, spiral or coccoid, and several studies have shown that the coccoid form cannot be cultured or be detected by RUT or histology but can be detected PCR (Bode *et al.*, 1993; Chan *et al.*, 1994; Hulten *et al.*, 1996; Moayyedi and Dixon, 1998; He *et al.*, 2002). Consequently, another possible explanation for our findings could be a high percentage of the coccoid form of *H. pylori* as a result of antibiotic treatment in the patients from group B. Additionally, He *et al.* (2002) have suggested the possibility that no viable *H. pylori* were present in the biopsy sample but only dead bacteria or chromosomal DNA. However, we do not know how long the bacteria or the DNA can remain in the stomach.

Several studies have shown that the eradication of *H. pylori* plays an important role in the treatment of some gastrointestinal diseases (Tytgat, 1994) and the results of all

currently used tests for bacterial detection can be affected by the treatment (Leodolter *et al.*, 2001; Vaira *et al.*, 2002). In conclusion, our findings show that the real-time PCR assay allows accurate and fast quantification of *H. pylori* DNA from gastric samples and should also be used to monitor treatment.

Acknowledgments

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