Detection of Helicobacter pylori in saliva and esophagus

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INTRODUCTION

Helicobacter pylori is a gastrointestinal pathogen, one of the causative agents of gastritis and peptic ulcer diseases. It has also been described as a risk factor for gastric carcinoma (Cover and Blaser, 2009). To date, how the microorganism is transmitted remains unclear and the main suggested route is person-to-person transfer by oral-oral and faecal-oral mode (Parsonnet et al., 1999). The natural habitat for the microorganism is the human stomach, but it may also survive in other environments, such as dental plaque (Dowsett and Kowolik, 2003; Anand et al., 2006; Gebara et al., 2006; Souto and Colombo, 2008), human and animal faeces (Kabir 2003) and aquatic systems (Adams et al., 2003; Cellini et al., 2005). On the other hand, the high levels of prevalence of the microorganism, especially in undeveloped countries, suggest that several routes can contribute to the H. pylori infection in the human population (Azevedo et al., 2007).

The low number of H. pylori cells isolated from districts different from the gastric environment might be due both to the difficulty in culturing H. pylori from sites colonized by fast-growing bacteria and the presence of bacteria entering the viable but not culturable (VBNC) state in which the microorganism modifies its morphology from spiral to coccoid (spherical) form with a loss of cultivability (Cellini et al., 1998; Andersen and
Rasmussen, 2009). Consequently, biological samples such as saliva or feces may not allow the selective growth of a fastidious bacterium such as H. pylori requiring more appropriate techniques (Kabir 2003; Rasmussen et al., 2010; Wnuk et al., 2010). More suitable methodologies, such as new high PCR assays, provide a sensitivity in H. pylori detection in particular in over-crowded niches (Bamford et al., 1998; Kabir 2004; Cellini et al., 2008).

Some studies indicate the oral cavity as a transient or permanent location of H. pylori, in particular in patients with gingivitis or chronic periodontitis (Souto and Colombo, 2008). Moreover, the detection of H. pylori in the mouth can occur independently of stomach colonization (Bürgers et al., 2008) suggesting that the human oral cavity could represent an important extra-gastric reservoir for H. pylori (Suzuki et al., 2008; Kabir 2004). Also interesting is the exploration of other ecological niches for H. pylori from the oral cavity to the stomach such as the esophagus (Cellini et al., 2001; Bürgers et al., 2008). In this district, the controversial role of the presence of H. pylori is widely discussed in the literature (Suerbaum 2009; Rokkas et al., 2007) and the analysis of the microbiome of the distal esophagus has been considered an important approach to define healthy and diseased esophagus with or without H. pylori (Yang et al., 2009).

In a previous study (Cellini et al., 2001), we demonstrated the colonization of H. pylori throughout the gastrointestinal tract from esophagus to rectum of Balb/C mice persisting for at least 45 days. In this murine model, a high concentration of H. pylori was found in esophagus suggesting the latter a feasible reservoir for infection and transmission. The aim of our study was, therefore, to verify the presence of H. pylori in esophagus and saliva of humans.

**MATERIALS AND METHODS**

**Patients and study protocol**

Patients were identified among a group of individuals already treated unsuccessfully for H. pylori infection who were found positive at 13C-Urea Breath test (UBT) (AB ANALITICA srl, Padova, Italy) performed after one or more triple therapy for H. pylori infection. The patients underwent endoscopy because of a persistence of gastrointestinal disturbances, and to obtain a gastric biopsy specimen for culture and susceptibility test. From each patient, a biopsy specimen was taken from the antrum and fundus in the stomach and from the middle-distal esophagus approximately 5 and 7 cm above the gastro-esophageal junction. One milliliter of unstimulated whole saliva was also collected by expectoration into a sterile eppendorf tubes (Eppendorf s.r.l., Milano, Italy) for H. pylori molecular analysis. The saliva samples were collected before EGDS whereas esophagus samples were collected before the gastric examination. All specimens collected for molecular analysis were stored until DNA extraction. Gastric biopsies were used for H. pylori culture and antimicrobial susceptibility tests whereas saliva samples were collected to detect H. pylori with a Nested-PCR targeting 16S rRNA gene as well as esophagus biopsies which were also investigated with immunohistochemical staining.

Exclusion criteria were: age <18 or >80 years, use of proton pump inhibitors (PPIs) and antibiotics within the previous 4 weeks and severe concomitant diseases, previous gastric surgery, pregnancy or lactation, alcohol abuse, drug addiction, chronic use of corticosteroids or non-steroidal anti-inflammatory drugs, Barrett esophagus and esophagitis grade II and III following Los Angeles classification (Armstrong et al., 1996).

**H. pylori culture and antibiotic susceptibility tests**

Biopsy samples, from antrum and fundus, were collected for culture in Portagerm-Pylori (BioMérieux Italia S.p.A., Roma, Italy) and processed microbiologically within 24 h. Biopsies were trimmed with a razor, homogenized and cultured on Chocolate agar plus 1% IsoVitaleX (CA, BD Becton Dickinson Italia S.p.A., Milano, Italy) and Campylobacter selective medium (CP, Oxoid Limited, Basingstoke Hampshire, UK). Plates were incubated under a microaerophilic atmosphere at 37°C for 5-7 days. In negative cases, an additional incubation of 7 days was carried out to recover slow growing cells. H. pylori colonies were identified on the basis of their colony morphology, Gram staining and positive reaction with urease, catalase and oxidase. Isolated strains were stored at –80°C using the Drumm and Sherman method (1989).
For the antimicrobial susceptibility tests, antibiotic concentrations were used following the cut-off levels related to the breakpoint for Amoxicillin (Sigma, Milan, Italy), Clarithromycin (Abbott Laboratories, North Chicago, IL, USA), Levofloxacin (FLUKA-Biochemica, Buchs, Switzerland), Moxifloxacin (FLUKA-Biochemica), Rifabutine (Pharmacia & Upjohn, Ascoli Piceno, Italy), and Tinidazole (Sigma) (Andrews et al., 1999; Toracchio et al., 2000; Aydemir et al., 2005; Toracchio et al., 2005). Powders were reconstituted following the manufacturer’s instructions; blood agar media consisting of Mueller-Hinton agar (Bioline Italiana, Milan, Italy) plus 7% (v/v) of lacked horse blood was added to 5 µg/ml of Tinidazole, 1 µg/ml of Clarithromycin, 0.5 µg/ml of Amoxicillin, 0.05 µg/ml of Rifabutine, 5 µg/ml of Levofloxacin and 5 µg/ml of Moxifloxacin respectively (CLSI 2006). Spread plates were inoculated in a microaerophilic atmosphere at 37°C for 3-5 days. All tests were performed in triplicate.

Immunohistochemical method

Immunohistochemical staining of esophagus samples was performed on 5 mm thick paraffin sections. After proteolytic predigestion with proteinase K, sections were tested with the specific antibody Dako rabbit anti-H. pylori (dilution 1:25) in Dako diluent (Glostrup, Denmark). After further incubation with biotinylated anti-rabbit immunoglobulin and washing in Tris-buffered saline, the standard avidin-biotin peroxidase complex method was applied. The rabbit anti-H. pylori specific polyclonal antibody raised against heat-stable antigens of H. pylori strain CH-20429 is highly specific for H. pylori (Ashton-Key et al., 1996) and has a sensitivity of 100% and specificity of 94% in distinguishing H. pylori from other curved bacteria present in the tissues (Andersen et al., 1988). Heat-stable antigens of H. pylori were responsible for a specific humoral immune response in patients infected by this strain (Andersen et al., 1992). Tissue sections were evaluated blindly by three pathologists unaware of the experimental procedure.

Nested-PCR amplification and DNA sequencing

DNA was extracted directly from each esophagus biopsy and saliva samples with QIAamp Tissue DNA isolation Minikit (QIAGEN, S.p.A., Milan, Italy) and used as template for the specific detection of H. pylori 16S rRNA gene. For 16S rRNA gene detection, a nested-PCR was performed and a 109 bp DNA fragment was amplified with oligonucleotide primers Hp1 (5’- CTG GAG AGA CTA AGC CCT CC - 3’), Hp2 (5’- ATT ACT GAC GCT GAT TGT GC - 3’) and Hp3 (5’- AGG ATG AAG GTT TAA GGA TT - 3’) (Applied Biosystem, Monza, Italy) according to Ho et al. (1991). Two µl of extracted DNA of each sample were added to a final volume of 25 µl of reaction mixture containing 10X PCR buffer (50 mM KCl, 10 mM Tris HCl, pH 8.3), 3 mM MgCl₂, 250 µM of deoxynucleotide triphosphate, 25 pmol of each primer (Hp1 and Hp3) and 1 U of Amplitaq DNA polymerase (Applied Biosystem, Monza, Italy). PCR amplification of Hp1-Hp3 was performed according to the following profile: 95°C for 5 min and 35 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, followed by 72°C for 5 min in an Applied Biosystem Thermocycler 2700.

After PCR, 2 µl of the final product was transferred in a second step reaction mixture and re-amplified for 35 cycles with the Hp1 and Hp2 primers using the same PCR conditions and amplification program except for the annealing temperature corresponding to 62°C. In all analyses, H. pylori ATCC 43629 was used as positive control and E. coli ATCC 25992 as negative control; an esophagus biopsy and saliva sample of a patient UBT negative were also included in the examinations. Six µl samples of PCR products were analyzed by electrophoresis on a 2% (w/v) agarose gel at 100 V for 1 h. Gels were stained with ethidium bromide and photographed.

The 16S rRNA PCR products were purified by spin column QIAquick (QIAGEN) and cycle-sequenced (on both strands) by using the ABI PRISM Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). DNA sequences were analysed on an automated sequencer, ABI PRISM 310, version 3.4.1 (Applied Biosystems). The resulting nucleotide sequence of the 109 bp region of the 16S rRNA gene was aligned using the Sequence Navigator software package (Applied Biosystem). Sequence comparison was subsequently carried out using BLAST Search in National Center of Biotechnology Information (NCBI).
RESULTS AND DISCUSSION

Nineteen patients (12 females and 7 males, mean age 46 years, range 22-77 years) were studied. Endoscopic findings included 7 patients with terminal esophagitis (grade I and II), 10 patients with antral erosive gastritis and 2 patients with normal findings.

From the 19 UBT positive patients examined, *H. pylori* was isolated by culture in 18 out of 19 subjects both in antrum and fundus, and each examined sample was studied for the susceptibility against the most common antibiotics used in the anti-*H. pylori* therapy. Figure 1 shows the percentage values of resistance recorded in microorganisms coming from gastric antrum and fundus. In particular, Clarithromycin was ineffective against 72.2% and 61.1% of patients positive for *H. pylori* isolated in antrum and fundus, respectively, while Tindazole was ineffective against 61.1% and 50% of patients positive for *H. pylori* in gastric antrum and fundus, respectively. In two cases, corresponding to patients with antral erosive gastritis, the susceptibility patterns were different from antrum and fundus suggesting a multiple *H. pylori* infection. These data emphasize the need to perform an antimicrobial agents susceptibility test in more than one biopsy sample to plan a correct anti-*H. pylori* therapy.

With regard to the molecular analysis of esophageal biopsies and saliva samples, all the Nested-PCR targeting the 16S rRNA gene, gave positive results including the patient with a negative culture in antrum and fundus. Figure 2a shows a representative electrophoresis gel of an esophagus and saliva sample with a clear amplification of a fragment of 109 bp. The Nested-PCR of the samples coming from the esophageal biopsy and saliva sample of the patient UBT negative do not display any amplification (not shown). An esophagus biopsy and saliva sample of a patient UBT negative were also included in the examinations. Each positive sample detected both in the esophagus and in the saliva was confirmed to be *H. pylori* by the comparative sequence analysis. The comparison between the alignments of the 16S rRNA ORFs of each esophageous and saliva sample and *H. pylori* J99 always gave an identity ≥ of 98%. A representative couple of sequences (patient number 48) coming from esophageous and saliva samples (48E/48S) were deposited in the NCBI database (Genbank accession numbers: GU725436; GU725435). Although several authors reported the presence of *H. pylori* in saliva and in esophageous (Quiding-Jarbrink *et al.*, 2009; Suzuki *et al.*, 2008) this study shows for the first time that, in patients with gastric infection with *H. pylori*, the *H. pylori* DNA is detectable in the saliva and the esophagus as well.

When the esophageus samples were detected by immunohistochemical staining, the presence of heat-stable antigens of *H. pylori* was detected in 3 out 19 biopsies evaluated. Figure 2b displays a representative case of *H. pylori* positive sample coming from esophageal tissue. The microscopic appearance of area of mucosa with positive *H. pylori* reaction was characterized by coccoid aggregated bacteria. On the other hand, it is well known that *H. pylori* is able to modify itself from spiral to coccoid form in a clustered altruistic state challenging the environmental stress due to an unsuitable niche (Cellini *et al.*, 2005; Burges...
et al., 2008). However, coccoid *H. pylori* preserves its intact membrane structure which has a strong stainability with *H. pylori*-specific antibodies (Saito et al., 2003). In our study, in the esophagus, the immunohistochemical staining confirms its specificity in identifying *H. pylori* cells in a district containing a lot of Gram negative and Gram positive taxa, but it results less susceptible than molecular method (Suerbaum 2009; Yang et al., 2009). The possible presence of coccoid bacteria embedded in a biofilm matrix could interfere with the specific link of the antibody to the surface of the microorganism, giving false negative results. On the contrary, the molecular methods (nested PCR and sequencing) are both specific and sensitive despite the presence of biofilm since the DNA purification could break up the matrix.

Indeed *H. pylori* has been found in the columnar-lined epithelium in patients with Barrett’s esophagus (BE) (Newton et al., 1997) a disease that was excluded in our patients. Several bacteria other than *H. pylori* were also identified from esophageal mucosal biopsies taken from patients with normal esophagus (Macfarlane et al., 2007).

FIGURE 2 - Representative images of *Helicobacter pylori* positivity in esophagus and saliva. a) Agarose gel electrophoresis of Nested-PCR of *H. pylori* 16S rRNA gene; lane 1: saliva sample; lane 2: negative control (Escherichia coli ATCC 25922 DNA); lane 3: positive control (*H. pylori* ATCC 43629 DNA); lane 4: esophagus sample. M indicates the DNA molecular weight marker (0.1 Kbp, Sharpmass™, EuroClone, Pavia, Italy). The results presented here are representative of those obtained from two independent experiments with three replicates. b) Immunoperoxidase staining of esophagus sample. The arrow indicates positive immunoreactivity for *H. pylori*. Original magnification, x 400.

These bacteria were often found to be growing in microcolonies and cell aggregates, such as *H. pylori* found in the present study. The PCR and immunostaining study allows us to identify *H. pylori* in these specimens.

The results of the present paper show that the *H. pylori* DNA was detectable in saliva and esophagus in 100% of the 19 patients examined. The analysis for the specific detection of a highly conserved region of *H. pylori* 16S rRNA gene (Ho et al., 1991) through Nested-PCR improved sensitivity in *H. pylori* identification in samples containing both prokaryotic and eukaryotic cells as well as many organic impurities (Bamford et al., 1998). Thus, Nested-PCR appears to be the best system in *H. pylori* detection in saliva and esophagus (Kabir 2004; Rasmussen et al., 2010). Moreover, the specificity of the molecular methodology used in this study was also confirmed by the sequencing PCR amplicons that represents the crucial test to ascertain the presence of *H. pylori*, in particular, in samples colonized by other bacteria (Medina et al., 2009; Kabir 2004). However, as well reported in the literature, the results depend widely on the detection
method chosen as well as by the morphological or aggregative status (VBNC and/or biofilm) of bacteria. For this reason, continued improvements to current tests and the development of new tests is desirable to avoid false negative results and prevent the occurrence of severe gastric diseases. We found \textit{H. pylori} in saliva and esophagus also in a patient with negative culture in antrum and fundus. At present we do not know how often \textit{H. pylori} remains and for how long in the saliva and in the esophagus after eradication from the stomach and whether its may be source not only of transmission, re-infection or esophageal disease. Further studies are necessary to clarify this issue.

The colonization of humans by \textit{H. pylori} is a complex process and probably several routes contribute to the prevalence levels of \textit{H. pylori} in the population (Adams et al., 2003; Cellini et al., 2005; Azevedo et al., 2007). Certainly the main responsible route for \textit{H. pylori} transmission is direct person-to-person (oral-oral, gastro-oral) transmission and the use of more susceptible techniques for \textit{H. pylori} detection, in particular in overcrowded sites, can contribute in \textit{H. pylori} localization also identifying the presence of the microorganism in organized microbial communities such as bacterial biofilm (Azevedo et al., 2007; Cellini et al., 2008; Suerbaum 2009). The understanding of the possible route of \textit{H. pylori} transmission is crucial in developing public health measures of surveillance by finding new means of disease management.

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not culturable state: regrowth or resuscitation? 

**H. pylori in saliva and esophagus**

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