

Detection of *Helicobacter pylori* in saliva and esophagus

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SUMMARY

The route of *Helicobacter pylori* transmission remains unclear and the currently suggested route is person-to-person transfer by faecal-oral and oral-oral mode.

The aim of this study was to verify the presence of *H. pylori* in esophagus and saliva of humans. Saliva samples, mucosal biopsies from esophagus, gastric antrum and fundus were collected from 19 patients with positive Urea Breath Test (UBT).

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.

Helicobacter pylori was isolated in 18 patients both in gastric antrum and fundus. The molecular analysis, confirmed by comparative sequences evaluation, gave positive results in all saliva and esophageal samples whereas the immunohistochemistry revealed the presence of *H. pylori* in 15.8% (3/19) of the esophagus samples.

Our data suggest that saliva and esophagus may be considered reservoirs for *H. pylori* in humans and emphasize the need to use more susceptible techniques for *H. pylori* detection, in particular in over-crowded sites. Identification of the transmission route of *H. pylori* is crucial in developing an effective plan of surveillance by finding new means of disease management.

KEY WORDS: *Helicobacter pylori* transmission, Esophagus, Saliva, Nested-PCR

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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

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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 a 13C-Urea Breath test (UBT) (AB ANALITICA srl, Padova, Italy) performed after one or more triple therapy for *H. pylori* infection. The patients underwent en-

doscopy 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 (Biolife Italiana, Milan, Italy) plus 7% (v/v) of laked 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 incubated 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 Tinidazole 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. In particular, the test should be performed in patients already treated for *H. pylori* infection, in whom the percentage of multidrug resistant strains is higher than in patients with primary infection

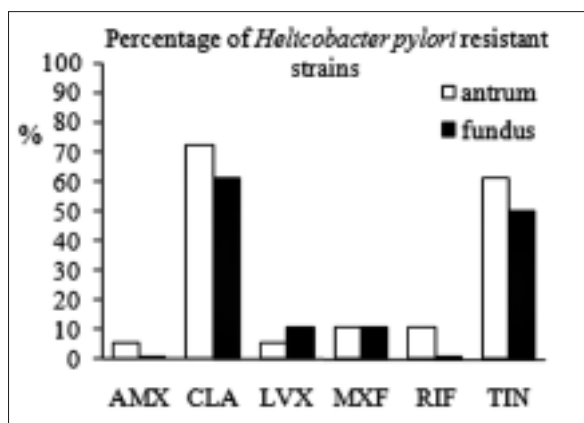


FIGURE 1 - Percentage of *Helicobacter pylori* resistant strains isolated from gastric antrum and fundus of 18 patients already treated for *H. pylori* infection.

(Toracchio *et al.*, 2000; Toracchio *et al.*, 2005). Moreover, the worrying problem of Clarithromycin ineffectiveness found in Italy was confirmed particularly in our geographic area (Toracchio *et al.*, 2000; Toracchio *et al.*, 2005; Gisbert *et al.*, 2010). Interestingly, the 2 patients with normal findings, harbored in antrum and fundus, *H. pylori* was susceptible to the antimicrobial agents tested.

With regard to the molecular analysis of esophagus 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 esophagus 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 esophagus and saliva sample and *H. pylori* J99 always gave an identity \geq 98%. A representative couple of sequences (patient number 48) coming from esophagus 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 esophagus (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 esophagus samples were detected by immunohistochemical staining, the presence of heat-stable antigens of *H. pylori* was detected in 3 out of 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

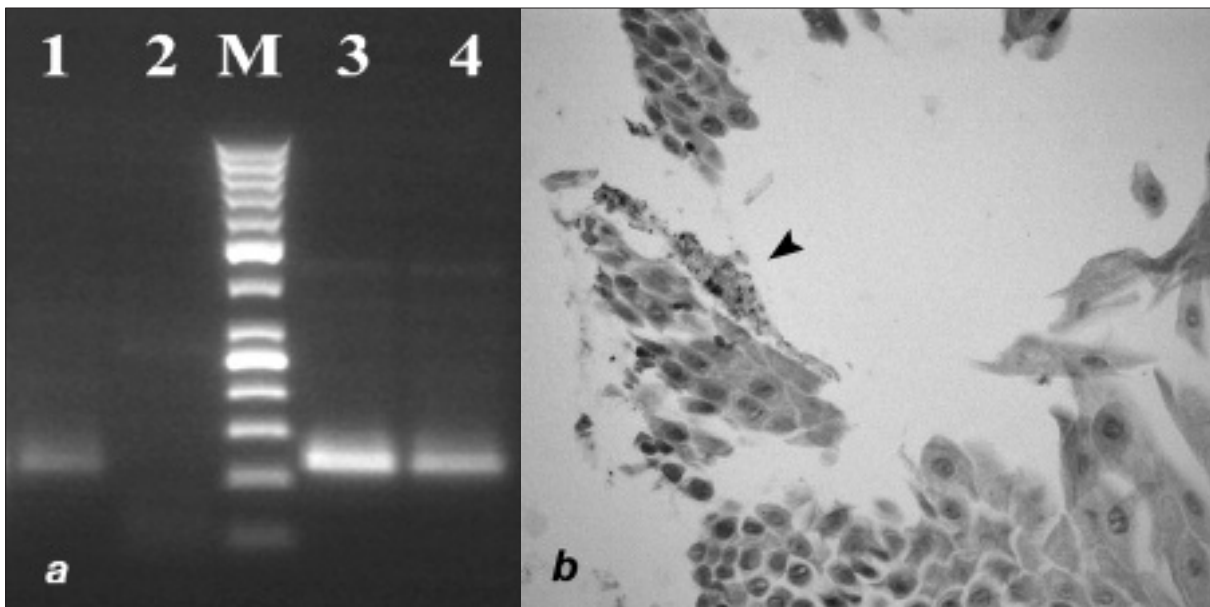


FIGURE 2 - Representative images of *Helicobacter pylori* positivity in esophagus and saliva. a) Agarose gel electrophoresis of Nested-PCR of *H. pylori* 16S rRNA gene; lane 1: saliva sample; lane 2: negative control (*Escherichia coli* ATCC 25992 DNA); lane 3: positive control (*H. pylori* ATCC 43629 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.

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).

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 *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 *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 *H. pylori* is a complex process and probably several routes contribute to the prevalence levels of *H. pylori* in the population (Adams *et al.*, 2003; Cellini *et al.*, 2005; Azevedo *et al.*, 2007). Certainly the main responsible route for *H. pylori* transmission is direct person-to-person (oral-oral, gastro-oral) transmission and the use of more susceptible techniques for *H. pylori* detection, in particular in overcrowded sites, can contribute in *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 *H. pylori* transmission is crucial in developing public health measures of surveillance by finding new means of disease management.

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