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Short communication

Absence of *Helicobacter pylori* in the oral cavity of 10 non-dyspeptic subjects demonstrated by real-time polymerase chain reaction

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Helicobacter pylori plays a significant role in gastric disease. However, the presence of this bacterium in the oral cavity remains controversial. The aim of the present study was to detect and quantify *H. pylori* in 29 different sites of the oral cavity in non-dyspeptic subjects by means of real-time polymerase chain reactions (PCR). Ten subjects without gastric symptoms were studied. Samples from unstimulated saliva, three sites of the tongue, oral mucosa, and 12 sites of both supragingival and subgingival plaque were collected from each subject. DNA was extracted from the oral samples and analysed for the presence of *H. pylori* by real-time PCR (LightCycler®) using JW23/22 primers which targeted the 16S rRNA gene. DNA from *H. pylori* DSM 4867 was used as a positive control. Amplification efficiency for the LightCycler® 2.0 runs ranged from 1.8 to 2.4. Melting curve analysis identified all the positive control capillaries, which contained *H. pylori* reference DNA, as a single and narrow peak at a melting temperature between 84.5 and 84.9°C. All the negative control capillaries with no template control and the 29 oral samples from each subject showed either no melting peaks or broad melting peaks below 80°C, which were considered as primer dimers. Therefore, *H. pylori* was not detected from any of the 290 oral samples. As a conclusion, *H. pylori* seems not to be permanently present in the oral cavity of a non-dyspeptic population.

Key words: dental plaque; *Helicobacter pylori*; mucosa; real-time polymerase chain reaction; saliva; tongue

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Helicobacter pylori is a gram-negative, curved, microaerophilic organism that has been implicated in the aetiology of gastritis, in the process of gastric and duodenal ulcer formation, and in gastric cancer (11, 13). Approximately 10% of individuals are affected by gastritis and/or gastric ulcer during their lifetime and over 50% of the world's population carries this infection (6). The mode of transmission of *H. pylori* is poorly understood, although the oral–oral, gastric–oral and faecal–oral routes are all possible. The natural reservoir for *H. pylori* is unknown. A literature review failed to find evidence supporting the role of the oral cavity as a significant reservoir of *H. pylori* (6, 10, 16). If the oral cavity is a reservoir for *H. pylori*, it is unclear whether it is a permanent or a transient reservoir and its specific niche has not yet been identified (6).

Polymerase chain-reaction (PCR) allows rapid detection of even small numbers of specific bacteria within a sample. By this method, *H. pylori* has been detected in oral samples, but results show a great variation as the published reviews point out (6, 10, 16). Recent studies using conventional PCR still report very variable results, with a detection rate ranging from 0% to 100% (1, 4, 7, 8, 17, 22–23). These discrepancies can be explained by differences in the study populations, oral sample collection methods and laboratory detection procedures. Therefore, the most appropriate method for detection of oral *H. pylori* has yet to be established (6). Real-time PCR has proved to be the most accurate method for *H. pylori* detection in gastric biopsies because of its high specificity, short working time, low risk of contamination, and the fact that it yields a quantitative analysis (20). To our knowledge no study has used real-time PCR to detect *H. pylori* in the oral cavity. Hence, the aim of the present study was to investigate the presence of *H. pylori* at 29 different sites in the oral cavities of 10 healthy subjects, using real-time PCR (LightCycler® 2.0).

Ten subjects from the Dental School of Toulouse University (France) without gastric symptoms (five men aged 27–53 years, mean age 36.2, and five women aged 26–38 years, mean age 30.4) were studied. The subjects presented neither gingivitis nor periodontal disease and had not used any kind of oral rinses and/or systemic drugs within the previous 4 weeks. All were informed of the study and each signed the informed consent form approved by the local ethics committee.

Subjects were instructed to avoid oral hygiene in the morning and did not smoke. Unstimulated saliva samples from each subject were collected in sterile Falcon polypropylene tubes. Four oral mucosa samples was taken from dorsum of tongue, right and left lateral tongue, lingual tonsil, and right and left cheek using a plain sterile swab (Copan®, Brescia, Italy). Twelve supragingival plaque samples were collected from first molars, first premolars and lateral incisors using a plain sterile swab (Copan®, Brescia, Italy). After collection of supragingival plaque, subjects were instructed to brush their teeth to avoid supragingival plaque contamination. Afterwards, 12 subgingival plaque samples were collected from the same teeth as the supragingival samples by means of 4 sterile paper points left in place for 20 s. All samples were coded and analyses were performed blind.

H. pylori strain DSM 4867 (CIP 103995-T, ATCC 43504, NCTC 11637) was used as a reference strain. The bacteria were grown in brain–heart infusion agar supplemented with 5% (volume/volume) defibrinated sheep blood. Plates were incubated micro-aerobically (CampyPak®, BBL Microbiology systems, Cockeysville, MD, USA) at 37°C for 5–7 days. Colonies of *H. pylori* were identified by colony morphology, microscopy, Gram-stain and positive reactions for catalase, oxidase and urease. Genomic DNA was obtained from *H. pylori* culture and from oral samples by the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

The assay was performed in the LightCycler® 2.0 real-time PCR instrument (Roche Diagnostics GmbH) in the SYBR Green I format which was targeted at the 16S rRNA gene. The primers used were JW23/22 (7, 19): forward primer 5′-GAG CGC GTA GGC GGG ATA GTC-3′; nucleotides 536 to 556 in GenBank sequence accession no. U01330, reverse primer 5′-CGT TAG CTG CAT TAC TGG AGA-3′; nucleotides 830 to 810 in GenBank no. U01330 (Proligo® Primers & Probes, Paris, France). Each nucleotide sequence was evaluated on-line using the BLAST nucleotide algorithm to verify specificity. Serial 10-fold dilutions of DNA extracted from *H. pylori* strain DSM 4867 were used as standards to determine the efficiency of each assay and were included in every set of reactions as a positive control. Negative control reactions with no added template DNA were also used in each set of reactions. A titration experiment was performed using different primers and $MgCl_2$ concentrations. Samples were processed using a LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH) with 6.6 μl pure water, 2.4 μl $MgCl_2$ (at a final concentration of 4.0 mM), 2 μl of each primer (at final concentration of 0.8 μM), 2 μl Mix-Taq, and 5 μl DNA sample. The assay was run using the cycling conditions shown in Table 1.

Melting curves were plotted automatically and analysed with the LightCycler® 2.0 software. Since the melting temperature (T_m) of reference *H. pylori* DNA for the 16S rRNA assay was about 84.5–85°C in preliminary experiments, samples were considered positive for the presence of *H. pylori* DNA when a T_m of about 84–85.5°C was obtained. Samples exhibiting broader peaks with a T_m below 80°C were considered as primer dimers or non-specific products.

Amplification efficiencies for the LightCycler® 2.0 runs were very close to 2, ranging from 1.8 to 2.4, which implied a doubling of the amplification product at every cycle (Fig. 1). Melting curve analysis identified all the positive control capillaries, containing *H. pylori* reference DNA, as a single, narrow peak at a melting temperature between 84.6 and 84.9°C. All the negative control capillaries with no control template and the 29 oral samples from each subject showed either no melting peaks or broad melting peaks <80°C which were considered as primer dimers (Fig. 2). Therefore, the 16S rRNA gene of *H. pylori* was not detected in any of the 290 oral samples of non-dyspeptic subjects.

To exclude the possibility of PCR amplifications being negative due to the primer dimers, a second positive control was tested mixing 1 μl of *H. pylori* DNA reference strain in 4 μl of DNA extracted from one saliva and one dental plaque sample from each subject, and these 20 samples were analysed by real-time PCR. To know if the sensitivity for *H. pylori* decreases due the presence of inhibitors in saliva or dental plaque, a third positive control was tested adding *H. pylori* to two saliva samples and to two dental plaque

Table 1. Cycle programs and temperature profile used on the LightCycler instrument for the SYBR Green I format

Program	No. of cycles	Analysis mode	Segment	Target (°C)	Hold time	Slope (°C/s)	Acquisition mode
Denaturation	1	None		95	10:00	20	None
Amplification	45	Quantification	Denaturation	95	00:15	20	None
			Annealing	66	00:10	20	None
			Extension	73	00:25	20	Single
Melting curve	1	Melting curves	Denaturation	95	00:02	20	None
			Annealing	66	00:20	20	None
			Extension	95	00:00	0.1	Continuous
Cooling	1	None		40	01:30	20	None

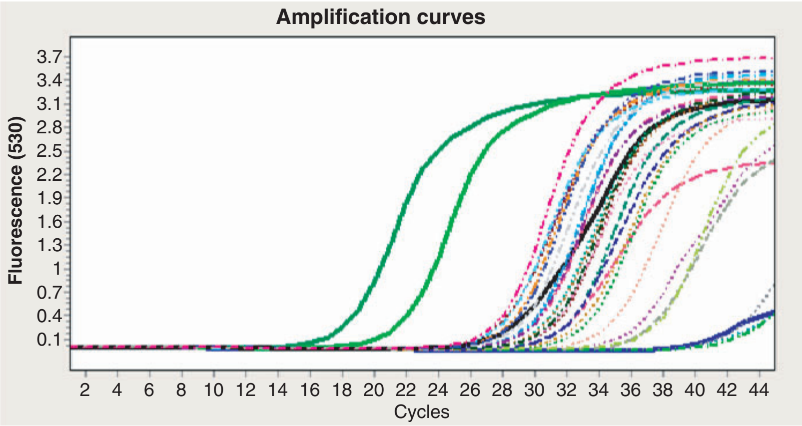

Fig. 1. Real-time PCR amplification of the two 10-fold dilutions of reference *Helicobacter pylori* DNA (green lines), negative control (blue line), and oral samples. The reaction was monitored with SYBR Green I.

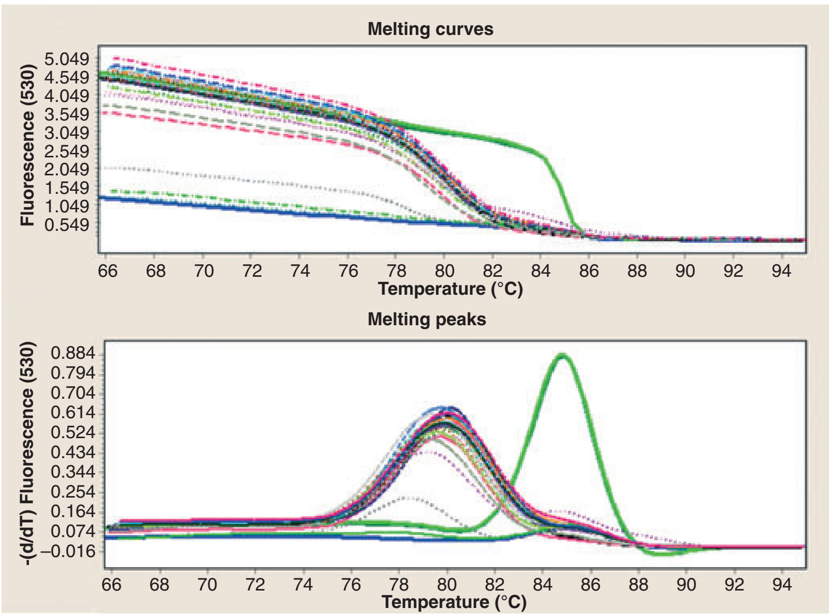

Fig. 2. Identification of 16S rRNA gene in *Helicobacter pylori* by real-time PCR melting curve analysis. The upper graph shows fluorescence versus temperature 'melting curves'. For the two 10-fold dilutions of reference *H. pylori* DNA (green lines), the SYBR Green I Dye fluorescence declined linearly with increasing temperature, followed by a steep decline in fluorescence as the specific product melted (around 85°C). The primer dimers and non-specific products produced in most of the oral samples melted at a much lower temperature. The lower graph shows the negative derivative of fluorescence with respect to temperature, converting melting curves into melting peaks. Melting temperatures of two 10-fold dilutions of reference *H. pylori* DNA (green lines) are between 84.6 and 84.9°C. Melting temperatures of negative control (blue line), and oral samples are <80°C and considered to be primer dimers.

samples and DNA was extracted and then analysed by real-time PCR. All of the positive controls showed amplification and a melting peak between 84.5 and 84.8°C.

Real-time PCR did not reveal *H. pylori* DNA in any oral sample from non-dyspeptic subjects. This is in agreement with other studies using conventional PCR in a non-dyspeptic population (3, 14, 17). Using conventional PCR, a great variation in the prevalence of oral *H. pylori* has been reported, probably as the result of large differences in study populations, oral sample collection, and laboratory detection procedures (6).

The population of the present study was young, lived in a developed country, and was without periodontal disease or gastric symptoms. If there were a relationship between oral and gastric *H. pylori* infection, one would expect older people, living in developing countries and having gastric symptoms to have higher oral *H. pylori* prevalence. Using conventional PCR, no clear association between age, country, periodontal disease and gastric symptoms with prevalence of oral *H. pylori* was observed in various studies (3, 5, 6, 7, 10, 16, 17, 19, 23). Therefore, comparisons of the prevalence and quantity of oral *H. pylori* between subjects with and without gastric symptoms, with and without periodontal disease, and in developing and developed countries could be the subjects of future research using a real-time PCR.

If the oral cavity were a permanent reservoir for *H. pylori*, a specific niche would exist. Therefore the type and the site of oral sampling are likely to influence the prevalence found for oral *H. pylori*. Whereas in some studies *H. pylori* was found to be more prevalent in dental plaque than saliva or oral mucosa (2, 7, 8, 18), others reported the same or a smaller percentage in dental plaque and saliva or oral mucosa (12, 14, 15, 23). This random distribution within various ecological niches is more consistent with occasional gastric reflux than with permanent oral colonization (10) and it is likely that *H. pylori* is a transient member of the oral microflora of specific populations (24). Longitudinal studies are needed to investigate the behaviour of this bacterium in the oral cavity over time.

The laboratory procedures play an important role in the sensitivity and specificity of PCR detection of *H. pylori* (6). Melting curve analysis performed by LightCycler® 2.0 resolves PCR products by GC content and length, whereas electrophoresis separates only by length. Therefore real-time PCR might have more specificity than conventional PCR. The possibility exists that the primers used in the studies that found a high oral *H. pylori* prevalence amplified the DNA of another bacterium. The choice of the primers is crucial to the sensitivity and specificity of the detection of *H. pylori* both in gastric samples (9) and oral samples (21). In the present study, primer JW23/22 was chosen to target the 16S rRNA gene, which exhibited a full sensitivity for *H. pylori* DNA reference strain, for *H. pylori* DNA mixed with DNA extracted from saliva or dental plaque, and for *H. pylori* added to saliva or dental plaque and has been used successfully by other authors (7, 19). Therefore, a decrease in the sensitivity produced by enzymatic inhibitors and/or the presence of primer dimers seems unlikely. Moreover, the BLAST search for the nucleotide sequence showed an extreme specificity for several strains of *Helicobacter*. This fact was verified in our laboratory in a preliminary study, which showed no reactivity of these primers with the DNA of three bacteria (*Bacillus stearothermophilus*, *Escherichia coli* and *Streptococcus mutans*).

Although the sample size in the present study was small, *H. pylori* does not seem to be permanently present in the oral cavity of the non-dyspeptic population. Further studies are needed to evaluate the prevalence of *H. pylori* in the oral cavity of subjects with gastric symptoms and/or periodontal disease, using real-time PCR.

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