Is *Helicobacter pylori* resident or transient in the human oral cavity?

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*Helicobacter pylori* colonizes the stomachs of at least half of the world’s human population. The role of the oral cavity in this colonization is not clear and there are, to date, no comprehensive data that clearly demonstrate the isolation of this bacterium from the oral cavity. The aim of this study was to evaluate the prevalence of *H. pylori* in the oral cavity of 15 patients who tested positive for *H. pylori*. A comprehensive dental examination of all patients was conducted. Samples were taken from supragingival and subgingival plaque, saliva, periapical exudates and tongue swabs. All samples were taken before the application of antibiotics. A total of 163 oral samples were investigated by PCR using two different *H. pylori*-specific primer pairs. A PCR inhibition control using a modified plasmid was always included for the most specific primer pair. In addition, a culture technique was used to confirm PCR results. Despite a PCR detection limit of 10^5 bacteria ml^-1, out of 14 patients, *H. pylori* could not be detected in any of the samples taken. In one patient, *H. pylori*-positive PCR signals were obtained in two samples using only one primer pair. *H. pylori* could not be cultivated from these two PCR-positive samples; therefore, no correlation to oral colonization status could be established. This study challenges the misleading preconception that *H. pylori* resides in the human oral cavity and suggests that this bacterium should be considered transient and independent of the oral status. To date, positive PCR results for *H. pylori* in the oral cavity have been overestimated and not critically interpreted in literature.
*Helicobacter pylori* is believed to be a member of the oral microbial flora and is discussed as being a late colonizer of the supragingival plaque (Rickard et al., 2003). On the other hand, due to the high sensitivity of PCR, the absence of *H. pylori* DNA in the different compartments of the oral cavity means an absence of the bacterium itself. However, this can only be confirmed if a PCR inhibition control is conducted simultaneously. Such a PCR inhibition control was not conducted in most of the studies published, to date, on the prevalence of *H. pylori* in the oral cavity.

In the present study, the prevalence of *H. pylori* in the oral cavity of 15 *H. pylori*-positive patients was evaluated. After comprehensive dental examination of each of the patients, as conducted by an experienced dentist, samples taken from supragingival and subgingival plaque, saliva, periapical exudates and tongue swabs were tested by specific PCR and nested PCR. A PCR inhibition control using a modified plasmid was designed and added to all of the PCRs. Two different primer pairs were used. Furthermore, PCR-positive results were evaluated by using culture techniques. The following results challenge the notion that *H. pylori* can reside within the oral cavity.

**METHODS**

**Subjects and samples.** A total of 15 *H. pylori*-positive patients (mean age 53.5 years) were involved in this study. All patients were suffering from chronic spontaneous urticaria and were randomly singled out during the diagnostic work-up searching for trigger foci. *H. pylori* infection was confirmed by an *H. pylori* monoclonal stool antigen test. A comprehensive oral examination was conducted by the same experienced periodontist. The oral examination included the following assessments: (i) dental status, number of teeth and implants, insufficient fillings/crowns, caries; (ii) pocket probing depth (PPD), gingival recessions (PCPUNC15, Hu-Friedy); (iii) bleeding on probing (BOP); (iv) assessment of furcation involvement (Hamp et al., 1975), grade 1 = up to 3 mm, grade 2 = more than 3 mm, grade 3 = completely passing with a naber probe (PQ2N, Hu-Friedy); (v) graduation of tooth mobility (Lindhe & Nyman, 1977), grade 1 = up to 1 mm horizontal, grade 2 = more than 1 mm horizontal, grade 3 = vertical or horizontal with the tongue or lip; (vi) plaque control record (O’Leary et al., 1972), teeth were examined for the presence of plaque at six sites per tooth after staining the plaque with a plaque disclosing solution (Mira-2-ton, Hager & Werken) and the index was documented as the percentage of plaque-positive sites; and (vii) sulcus bleeding index (SBI) (Mühlemann & Son, 1971), the gingiva was evaluated by slightly sounding the gingival margin at six sites per tooth and the mean of all values was taken. A minimal score of 0 (no gingivitis) up to a maximal score of 5 (severe gingivitis) was obtained.

Supragingival and subgingival plaque was collected with a sterile curette (Hu-Friedy) and subgingival exudate was collected with three sterile paper points (Roecko 45, Coltène-Whaledent) per site, which were kept in the sulcus for 10–20 s. Smears from the tongue were obtained with a sterile metal scraper.

All samples were taken at different sites each and were transferred to a vial containing 0.75 ml reduced transport fluid (Seyd & Loesche, 1972) and stored at −80°C until the microbial analysis was conducted.

**PCR detection.** DNA was extracted using the Qiamp DNA kit (Qiagen) according to the manufacturer’s instructions. For PCR detection the primers EHC-U (5′-CCCTCAGCGCATACG-TCCCAAAA-3′) and EHC-L (5′-AAAGAATCATAAAAAGGCCCAAAAC-3′), covering the region from 80076 to 80492 bp (417 bp product) of the *H. pylori* genome, were used. These primers were previously evaluated and found to be the most appropriate, concerning specificity and sensitivity (Sugimoto et al., 2009). PCRs were carried out in final volumes of 50 μl, which contained 5 μl PCR buffer, 1 μl dNTP mixture (0.2 mM each of dATP, dCTP, dGTP and dTTP; Peqlab), 25 pm each primer, 5 μl template DNA and 2.5 units Taq polymerase (Peqlab). The following conditions were used in the PCR: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 59°C for 45 s and extension at 72°C for 30 s, with a final extension step at 72°C for 5 min.

Each of the samples was also tested by nested PCR using the primers ET-5U (5′-GGGAAATCATAAAGGCGCAAA-3′) and ET-5UL (5′-TGAGACCTTTCTAAGACGCTCTGTT-3′). A 0.1 μl portion of the first PCR product served as a template for the second PCR. All other PCR conditions, including the temperature program, were the same as described earlier. Sterile water was used as a negative control. The positive control was DNA extracted from *H. pylori* (strain 26695). Additionally, the primers HP1 (5′-CTGGAGAGACTAACGTCCTCG-3′) and HP2 (5′-ATTACTGACGCTGATTGTGTC-3′) were also used for PCR detection, delivering a 109 bp-product. This primer pair was also evaluated by Sugimoto et al. (2009). The following conditions were used: initial denaturation at 94°C for 5 min; followed by 40 cycles of denaturation at 94°C for 60 s, annealing at 60°C for 60 s and extension at 72°C for 60 s, with a final extension step at 72°C for 10 min. Sterile water was used as a negative control.

The specificity of the PCR primers was examined using DNA extracted from 60 different strains belonging to the genera *Helicobacter* and *Campylobacter*, as well as other oral bacterial genera.

PCR products were analysed using electrophoresis in a 1.5% agarose gel in Tris/borate/EDTA running buffer (TBE), stained with ethidium bromide and visualized with UV light in an Alpha Imager HP (Innotech). All PCRs were performed at least twice. Positive results were confirmed by sequencing. In addition, a PCR inhibition control, prepared as described below, was conducted in parallel for each PCR.

**Construction of an inhibition control plasmid.** A synthetic construct was developed that contained an *Escherichia coli* gene sequence flanked by EHC-region primer sites and was cloned into pZero2 plasmid (Invitrogen) to be used as a positive amplification control. Therefore, the 413 bp EHC PCR fragment derived from amplification of the *H. pylori* strain G27 was cloned into the EcoRV restriction site of the pZero2 plasmid (Invitrogen), resulting in pZero2-EHC. The *E. coli* gene *fur* was amplified using the primers Fur-EC-LI (5′-CTCACTGATATTAAT3′) and Fur-EC-R1 (5′-AGTTGAGAGCTGTAAT3′). At this point, the 780 bp amplion was cloned into the DraI restriction site in the EHC region of plasmid pZero2-EHC, resulting in the pZero2-EHC-control plasmid. This inhibition control plasmid is depicted in Fig. 1.

**Culture technique.** Supragingival exudate and tongue smear samples from one patient were positive, as revealed by PCR. To isolate *H. pylori* from these two samples, serial dilutions of up to 10−7 were prepared. Different dilutions were plated on DENT agar (Oxoid), which is selective for the culturing of *H. pylori*. This agar contained vancomycin, trimethoprim, cefadolin, amphotericin B and tetraphenyltetrazolium chloride. The agar plates were incubated under microaerophilic conditions at 37°C for 5–10 days. Additionally, yeast–cysteine blood agar plates (HCB) and Columbia blood agar (CBA) plates were used. The CBA plates were incubated at 37°C in a 5–10% CO2 atmosphere for 3 days to cultivate facultatively anaerobic.
bacteria. The HCB agar plates were used to cultivate anaerobic bacteria at 37°C for 10 days in an anaerobic chamber (Anaerocult A; Merck). To activate and enrich putative H. pylori cultures, these dental probes were also cultivated in fluid culture medium BBF (Brucella broth) containing 5% fetal calf serum. After 24 h, 48 h and 6 days of incubation, 0.1 ml of the fluid culture was subcultured on DENT agar plates.

Colonies with a gold colouration suspected to be derived from H. pylori were subcultured to obtain pure colonies. To identify H. pylori, Gram-staining and biochemical tests for different enzymes, such as catalase, oxidase and urease, were conducted after subculturing of the suspected colonies.

RESULTS

The oral status and the occurrence of H. pylori in 15 patients previously tested as positive for H. pylori were determined. The oral status included evaluation of PPD, PI, BOP, SBI and carious teeth (Table 1). Additionally, the pH value of the saliva was determined for each volunteer. A total of 163 oral samples were investigated by PCR using two different H. pylori-specific primer pairs. To our knowledge, this is the first time a PCR inhibition control plasmid, constructed for the most appropriate primers, has been used in such a study. Furthermore, a culture technique was used

Table 1. The oral status of all patients

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Gender</th>
<th>Carious teeth</th>
<th>Insufficient restorations/caries (%)</th>
<th>PPD ≥ 4 mm (%)</th>
<th>PPD ≥ 4 mm (%)</th>
<th>BOP (%)</th>
<th>PI (%)</th>
<th>SBI</th>
<th>pH</th>
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<td>10.9</td>
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</tr>
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<td>36.8</td>
<td>12</td>
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<td>59.6</td>
<td>29.3</td>
<td>0.6400</td>
<td>7.40</td>
</tr>
<tr>
<td>5</td>
<td>61</td>
<td>M</td>
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<td>13.0</td>
<td>9</td>
<td>6.5</td>
<td>16.7</td>
<td>39.1</td>
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<td>45</td>
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<td>24.7</td>
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<tr>
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</table>
to isolate *H. pylori* from samples, which showed positive PCR results.

The oral status of the patients revealed that a mean 15.7% of the teeth examined were carious or insufficiently restored. A higher PI of 29.37–95.5% correlated with a higher percentage of carious or not sufficiently restored teeth. The PPD values showed that 10.5% of the measured teeth pockets correlated with an attachment loss. The mean BOP index and PI were 30.2 and 54.6%, respectively. These values for BOP and PI indicate cases of plaque-induced gingivitis or periodontitis. Two patients showed indications of periodontitis marginalis chronica and 11 patients had cases of local periodontitis. Only two patients showed no elevated PPD values. The SBI values were between 0.0062 and 1.83, which correlated with the BOP findings.

Using the primers EHC-U and EHC-L, none of the dental status parameters showed a correlation with infection with *H. pylori*. Despite a PCR detection limit of $10^2$ bacteria ml$^{-1}$, *H. pylori* could not be detected in any sample taken from 14 patients. In one patient with bad periodontal status and oral hygiene, as well as high levels of gingival inflammation, *H. pylori* was detected only in two sample types, periapical exudate and tongue swabs. *H. pylori* could not be cultivated from these two PCR-positive samples. As shown in Fig. 2, the PCR inhibition controls, conducted in parallel, confirmed the validity of all PCRs. The results of the PCR using primers EHC-U and EHC-L were confirmed by the use of primers HP1 and HP2, which revealed no positive PCR results (Fig. 3).

**DISCUSSION**

Due to the high infection rate of the world’s population, combined with the severe outcome of these infections in humans, the mode of transmission of *H. pylori* is still a major concern. The first contact between humans and *H. pylori*, leading to infection, takes place in the oral cavity. However, in the literature, it is evident that *H. pylori* is considered a typical member of the oral flora (Aas et al., 2005; Baumgartner et al., 2009; Namiot et al., 2010; Rickard et al., 2003). On the other hand, no cultivation of *H. pylori* has been reported from the oral cavity to date (Al-Ahmad et al., 2010). This point is very important in discussions concerning the transmission route, since, as a resident of the dental biofilm, this bacterium may represent a permanent source of transmission for infection. In the present study, the occurrence of *H. pylori* in different compartments of the oral cavity of *H. pylori*-positive patients was investigated, both by PCR and by confirmation of PCR-positive results using culture-dependent methods. In contrast with other studies conducted to date, an inhibition control plasmid was constructed in order to exclude false-negative results. This PCR inhibition control was included in all PCR tests. Furthermore, positive PCR results were confirmed by sequencing and further examination was carried out independently through the use of culture-based methods. It should be emphasized that most previous studies have not included sequencing and culture techniques to evaluate positive PCR results.

*H. pylori* could not be detected in most of the samples analysed in this study, even by PCR. As a result, a correlation between *H. pylori* prevalence and stomach infections or oral status could not be found in this study. *H. pylori* could not even be cultivated from the two PCR-positive oral samples.

As shown in Table 2, contradictory results have been presented in the literature concerning the occurrence of *H. pylori* in the oral cavity and the association of this bacterium with the oral health status of humans. Most published studies have aimed to investigate the oral cavity as a source for subsequent gastric infections, which, of course, is an important issue in this field of research (Namiot et al., 2010; Chaudhry et al., 2011; Silva et al., 2010; de Souza Gonçalves et al., 2009; Bago et al., 2011; Eskandari et al., 2010). In contrast to the results presented here, many studies clearly reported the occurrence of *H. pylori* in dental plaque and concluded that there was an association between gastroesophageal disease and dental oral status (Silva et al., 2009, 2010; Morales-Espinosa et al.,...
2009; de Souza Gonçalves et al., 2009). On the other hand, and in agreement with the results presented here, some authors have reported similar results, stressing that the oral cavity is not a reservoir for H. pylori in infected patients (Silva Rossi-Aguiar et al., 2009; Bürgers et al., 2008; Olivier et al., 2006).

It should be emphasized that, to our knowledge, all of the studies reported to date on the prevalence of H. pylori in the oral cavity were conducted using PCR, since no oral strains could be cultivated and isolated (Al-Ahmad et al., 2010). If PCR was not used, some authors concluded the occurrence of H. pylori in the oral cavity after detection of some antigens such as those revealed by the kit used for detection of antigens in stool samples (Namiot et al., 2010). Moreover, on the basis of PCR results, some authors have suggested that saliva and dental plaque may serve as a temporary reservoir for H. pylori in individuals with gastric disease (Silva et al., 2009). Even an antibacterial treatment therapy was recommended by some researchers on the basis of positive PCR results of H. pylori in oral samples. Andersen & Rasmussen (2009) stated that H. pylori could be detected in water supplies in its degenerative non-culturable dead form. Moreover, the authors reported that H. pylori is not able to reside and reproduce in the oral biofilm. This is in agreement with the results presented in this study where the results were confirmed using the inhibition control.

Both primer pairs used in this study showed a high sensitivity of <100 c.f.u. ml\(^{-1}\), which was also reported by Sugimoto et al. (2009). Only for two samples were the PCR results different between the two primer pairs. This could have been caused by small differences in the sensitivity. However, the second HP1/HP2 primer pair confirmed the results revealed by the culture technique. This sensitivity, in combination with using the PCR inhibition control, underlines the reliability of the data, suggesting an absence of H. pylori in the oral cavity of the patients involved in this study. However, Sugimoto et al. (2009) revealed false-positive results for PCR detection of H. pylori in clinical and environmental samples. The authors concluded that results of studies based on PCR identification of H. pylori in environmental samples should be viewed with caution. Based on this report, the prevalence of H. pylori in the oral cavity, as has been found in many studies to date, should be questioned since these studies were based entirely on PCR results. Additionally, in most studies showing negative H. pylori PCR results for the oral cavity, no PCR inhibition control was used. Since it is known that human saliva contains PCR inhibitors (Hedman et al., 2009), a higher number of oral samples could have revealed positive PCR results for H. pylori, detecting the DNA of this bacterium.
without any relation to \textit{H. pylori} prevalence in the oral cavity. Our results support the suggestions made by Sugimoto \textit{et al.} (2009). Furthermore, we speculate that \textit{H. pylori} cannot be resident in the oral cavity, but rather occurs only transiently in some of the mouth compartments such as the saliva and dental plaque. This is not contrary to the proposed transmission via the oral route, since saliva is continuously produced and swallowed in humans. This suggestion is in agreement with the results of Lindén \textit{et al.} (2008) who found that salivary mucins are likely to play a role during adhesion of \textit{H. pylori} in the oral cavity.

In conclusion, the occurrence of \textit{H. pylori} in the oral cavity does not correlate with an infected stomach or with the oral dental status of patients. Moreover, a misleading conviction that \textit{H. pylori} resides in dental plaque or in other oral compartments is the reason for a huge number of contradictory reports, which have included overestimation of PCR results. \textit{H. pylori} should be considered to be transiently present in the oral cavity. The survival time of \textit{H. pylori} in saliva and the oral cavity should be studied in order to increase knowledge regarding the transmission route of this pathogen.

\textbf{ACKNOWLEDGEMENTS}

This study was supported, in part, by the German Research Foundation (DFG, AL 1179/1-1). The authors thank Dr Marie Follo and Dr Anne Raffauf for the revision of the manuscript.

\textbf{REFERENCES}


