This is a pre- or post-print of an article published in
Rapid Communication

Live Helicobacter pylori in the root canal of endodontic-infected deciduous teeth

Christian Hirsch¹#, Nicole Tegtmeyer²#, Manfred Rohde³, Marion Rowland⁴, Omar A. Oyarzabal⁵, Steffen Backert²

¹Department of Paediatric Dentistry, University School of Dental Medicine, University of Leipzig, Leipzig, Germany
²School for Biomedical and Biomolecular Science, University College Dublin, Belfield Campus, Dublin, Ireland
³Department of Medical Microbiology, Helmholtz Centre for Infection Research, Braunschweig, Germany
⁴School for Medicine and Medical Science, University College Dublin, Belfield Campus, Dublin, Ireland
⁵Institute for Environmental Health, Inc., 15300 Bothell Way NE Lake Forest Park, Seattle, WA 98155, USA

#these authors contributed equally to this work

To whom correspondence should be addressed:
Christian Hirsch, University of Leipzig, University School of Dental Medicine, Department of Paediatric Dentistry, Nuernberger Straße 57, 04103 Leipzig, Germany;
E-mail: Christian.Hirsch@medizin.uni-leipzig.de;
Phone: #49 341 9721070, Fax #49 341 9721079, and

Steffen Backert, University College Dublin, UCD School of Biomolecular and Biomedical Sciences, Science Center West L231, Belfield Campus, Dublin 4, Ireland;
E-mail: Steffen.Backert@ucd.ie; Phone #353 1716 2155, Fax #353 17161183

Key words: deciduous teeth, electron microscopy, endodontics, Helicobacter pylori, protein profiling, RAPD fingerprinting, root canal
Abstract

Background Many PCR-based studies have shown that *Helicobacter pylori* DNA is prevalent in the oral cavity, but reports on the isolation of live bacteria are extremely rare. Thus, it is widely unclear if *H. pylori* can indeed survive in the oral environment.

Methods Here we used electron microscopy, selective growth techniques, urease assays, 16SrRNA PCR and Western blotting to investigate the possible presence of live *H. pylori* in 10 root canal and corresponding plaque samples of endodontic-infected deciduous teeth in three children.

Results Although *H. pylori* DNA was verifiable in several plaque and root canal samples by PCR, bacterial colonies could only be grown from two root canals, but not plaque. These colonies were unequivocally identified as *H. pylori* by microscopic, genetic and biochemical approaches.

Conclusions Thus, root canals of endodontic-infected teeth may be a reservoir for live *H. pylori* that could serve as a potential source for gastric re-infection.
Introduction

The International Agency for Research on Cancer characterized *Helicobacter pylori* as a type I carcinogen [1] that is responsible for gastritis, gastro-duodenal ulcers, and gastric malignancies in humans [2]. Although it is one of the most common infections in the world and known to be transmitted in early childhood, the exact route of transmission is still unclear [3]. In a recent meta-analysis, a close relationship between *H. pylori* infection in the oral cavity and stomach was found; the authors concluded that *H. pylori* in the oral cavity is more difficult to eradicate than in the stomach, and may therefore be a source of gastric re-infections. However, a specific populated niche in the oral environment is unknown [4].

The majority of the analyzed studies used specimens of dental plaque, saliva, or oral mucosa, and identified several *H. pylori* markers by various tests, such as the urea breath test, rapid urease test, *Campylobacter*-like organism test, or PCR. Although PCR studies have previously found *H. pylori* DNA in the oral cavity, reports of live *H. pylori* are extremely rare and highly inconclusive [4, 5]. Unequivocal identification of live *H. pylori* is only possible by direct culture, because erroneous PCR results can arise from transient *H. pylori* presence in the mouth via food, reflux of *H. pylori* or its DNA from the stomach to the mouth [6-8], or misclassification of other urease producing microorganisms. Thus, until now it is unclear if *H. pylori* can indeed survive in the oral environment. In this article we describe two case reports for the successful isolation of live *H. pylori* from the oral cavity, particularly from root canal samples of teeth.
Methods

Patient selection and characteristics

We selected three consecutive paediatric patients who received dental treatment under general anaesthesia because of severe early childhood caries. Table 1 summarizes data about the patients’ age and gender, as well as the tooth number for the extracted teeth. Altogether 10 teeth with pulp necrosis and chronic apical periodontitis were used for the analyses. The presence of gastric *H. pylori* in the children or their parents was not checked in the study, because there was no indication for gastric or abdominal problems. All parents gave their written consent for microbiological analyses of the teeth. The study protocol was reviewed and approved by the ethics committee of the University of Leipzig.

DNA isolation and *H. pylori* growth

Plaque and root canal samples were taken from each tooth. These samples were divided in three parts, one for conventional DNA isolation (DNA isolation kit, Qiagen, Hilden, Germany), a second for electron microscopy (see below), and a third for direct culturing. For culturing, the samples were incubated with 1 ml brain heart infusion medium by rigorous shaking at 200 rpm for 30 min, followed by growth on GC agar plates with 10% horse serum (containing 10 μg/ml vancomycin, 5 μg/ml trimetroprim, 10 μg/ml nystatin, and 10 μg/ml colistin) for 7 days at 37°C using the Campygen gas-generating system (all from Oxoid/Fisher Scientific, Dublin, Ireland) [9, 10]. Single bacterial colonies were further analysed and typical *H. pylori* strains (26695 and J99) were used as positive controls. To check for functional urease in *H. pylori*, the above GC agar plates were supplemented with Phenol red (100 μg/ml)
and urea (600 μg/ml) as described [11]. The molten was then acidified to pH 5 using 1M HCl [11].

16SrRNA gene PCR and electron microscopy

For PCR amplification of the 16S rRNA gene in the genus Helicobacter, primers 5’-AGA GTT TGA TYM TGG C-3’ and 5’-TAC GGY TAC CTT GTT ACG A-3’ were used, and amplicons were sequenced as described [10]. For field-emission scanning electron microscopy (FESEM), tooth samples were fixed in a sterile solution containing 5% formaldehyde, 2% glutaraldehyde in cacodylate buffer (0.1 mM cacodylate, 0.01 mM CaCl₂, 0.01 mM MgCl₂, 0.09 mM sucrose, pH 6.9). Samples were subsequently covered with an approximately 10 nm-thick gold film by sputter coating and examined in a field-emission scanning electron microscope using an Everhart Thornley SE detector and in-lens detector in a 50:50 ratio at an acceleration voltage of 5.0 kV as described [10].

Protein profiling and Western blotting

For protein profiling, pure plate-grown bacterial samples were run on 12% SDS-PAGE gels and analyzed by Coomassie blue staining or Western blotting [9]. The following primary antibodies were used: mouse monoclonal anti-CagA antibody (Austral Biologicals, San Ramon, CA, USA), mouse polyclonal anti-urease antibodies [9], and polyclonal rabbit antibodies recognizing a series of other H. pylori proteins. These antibodies were raised against peptides corresponding to the following conserved amino-acid (aa) residues in H. pylori strain 26695: BabA (aa 126–140: CGGNANGQESTSSTT), SabA (aa 172–186: CAMDQTTYDKMKKLA), OipA (aa 275–288: NYYSDDYGDKLDYK), NapA (aa 105–118: EFKELSNTAEKEGD), Slt (aa 492–505: LRRWLESSKRFKEK), HtrA (aa 90–
103:DKIKVTIPGSNKEY), FlaA (aa 93–106: KVKATQAAQDGQTT), VirB9 (aa 503–522: IKNYGELERVIKKLPLVRDK), VirB10/CagY (repeat region: VSRARNEKEKKE), and Cag3/Cagδ (aa 32–45: IKATKETKETKKEA). Rabbit anti-CagM, anti-CagN and anti-VacA antibodies were raised against the entire recombinant proteins. These antibodies were affinity-purified and prepared according to standard protocols by Biogenes GmbH (Berlin, Germany). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit polyvalent sheep immunoglobulin was used as secondary antibody (DAKO Denmark A/S, DK-2600 Glostrup, Denmark). Blots were developed with ECL Plus Western blot reagents (GE Healthcare, UK limited Amersham Place, UK) as described [9].
Results

To investigate if *H. pylori* was present in the 10 tooth samples, DNA isolated from both plaque and root canal samples was subjected to PCR to amplify a ∼1.5 kb DNA fragment derived from a 16S rRNA gene region that is highly conserved in *Helicobacter*. The expected strong PCR products were produced in two root canal and four plaque samples, suggesting that *H. pylori* DNA may be present in some but not all patients (Table 1). To isolate viable *H. pylori*, all samples were prepared and cultured for seven days on selective agar plates to suppress other bacteria. Single colonies were identified under microaerobic growth conditions in two of the 10 root canal samples (samples 4 and 5 from the patient #2), but not from any plaque sample. These two root canal samples were then subjected to FESEM investigation to see if typical *H. pylori* bacteria could be visualized. FESEM indeed revealed various *H. pylori*-like spiral-shaped organisms in the two samples in close association to teeth debris (Figure 1A, yellow arrows). These candidate *H. pylori* were approximately 0.2 µm in diameter and varied in length from 2–3 µm. Several monopolar flagella were also observed as it is typical of *H. pylori* [10]. In addition and as expected, coccoid bacteria of an unknown nature, which could also represent *H. pylori*, were observed (Figure 1A, blue arrows). These morphological data suggest the presence of live, spiral-shaped *H. pylori* in the root canal environment of teeth.

To exclude artifacts, bacteria were grown on selective acidified agar plates supplemented with urea, the substrate of *H. pylori* urease [11]. These analyses yielded functional urease enzymes allowing urea hydrolysis in root canal samples to high extent similar to *H. pylori* control strains, while retarded growth and no urea hydrolysis was seen in Δ*ureA* mutants or in all non-*H. pylori* samples from dental plaque (Figure 1B). To unquestionable identify *H. pylori*, we determined the 16S rRNA gene sequences from the two root canal isolates as described [10]. Both strains had completely identical sequences showing
strong homology to that of several published *H. pylori* strains (Figure 2A). To characterize our isolates further, we performed Western blotting and confirmed the presence of several well-known *H. pylori*-specific pathogenicity factors as compared to the fully sequenced strains 26695 and J99. Specific antibodies revealed the presence of urease subunits A and B as well as a major disease-associated factor, CagA (Figure 2B, arrows). In agreement with the observation of flagella by FESEM, we also found that our isolated root canal strains express the flagellin component FlaA (Figure 2B). Moreover, the presence of certain adhesins (BabA, SabA, and OipA), cag pathogenicity island encoded proteins (CagL, CagM, CagN, Cag3, VirB9 and VirB10) and other virulence factors (NapA, HtrA, Slt and VacA) could also be confirmed using specific antibodies by Western blotting (data not shown). Thus, our findings clearly indicate the successful isolation of live *H. pylori* from the root canal of teeth.
Discussion

*H. pylori* can be cultured from human stomach biopsies, but attempts to identify other natural reservoirs for these organisms or the routes by which they are transmitted to the stomach have been yet unsuccessful [5, 12]. Here, live bacteria from two root canal samples were unequivocally identified as *H. pylori*. To our knowledge, this is the first report of the recovery of viable *H. pylori* from root canal samples, suggesting that this environment may be a reservoir for survival and growth that could serve as a potential source for *H. pylori* transmission. It is possible that these bacteria are of gastric origin, and that patients carrying *H. pylori* in their dental root canals are also colonised by the same or different strains in the gastric mucosa. Colonisation of the root canal may explain why eradication is often unsuccessful as the antibiotic therapy used may not penetrate the root canal. Whether or not this environment represents a reservoir for *H. pylori* which facilitates transmission among humans is a pressing question for future studies.

Conflict of interest  The authors declare that they have no conflict of interest.
Acknowledgements

We thank Francisco Rivas Traverso for technical support. The work of S.B. is supported through grants by the German Science Foundation (Ba1671/8-1) and Science Foundation Ireland (UCD 09/IN.1/B2609).

References


*Helicobacter pylori* in dental plaque by reverse transcription-polymerase chain

8. Nguyen AM, El-Zaatari FA, Graham DY. *Helicobacter pylori* in the oral cavity. A

    Sewald N, König W, Backert S. *Helicobacter* exploits integrin for type IV secretion

10. Rivas Traverso F, Bohr URM, Oyarzabal OA, Rohde M, Clarici A, Wex T, Kuester D,
    Malfertheiner P, Fox JG, Backert S. Morphological, genetic and biochemical
    characterization of *Helicobacter magdeburgensis*, a novel species isolated from the

    Mittl PR, Benghezal M, Marshall BJ. Surface properties of *Helicobacter pylori* urease

12. Delport W, van der Merwe SW. The transmission of *Helicobacter pylori*: The effects
    of analysis method and study population on inference. Best Practice & Research
Figure Legends

Figure 1. Morphological analyses of two root-canal samples (4 and 5) by FESEM and urease tests. (A) FESEM revealed *H. pylori*-like spiral-shaped bacteria (yellow arrows) that were approximately 0.2 µm in diameter and varied in length from approximately 2–3 µm. Coccolid bacteria with 0.5–1 µm in diameter were also observed in large aggregates (blue arrows). Representative pictures are shown from two preparations. (B) Selection of bacteria producing functional urease on acidified agar supplemented with urea. Left samples: Root canal samples 4, 5 and strain 26695; the colour change from orange to red observed indicated that bacterial colonies were producing functional urease and growing. Right samples: 26695Δ*ureA*, plaque samples 4 and 5. Colour change did not occur on the right samples, indicating that functional urease was not being produced.

Figure 2. 16S rRNA sequencing and Western blotting analysis of *H. pylori*-specific pathogenicity factors. (A) Phylogenetic tree of 16S rRNA gene sequences of root canal samples and closest *H. pylori* strains. (B) Western blotting analysis of root canal samples for well-documented *H. pylori* proteins including ureaseA, ureaseB, CagA and flagellin A (FlaA).
Table 1. Sample Characteristics and *H. pylori* identification*

<table>
<thead>
<tr>
<th>Patient</th>
<th>#1, male</th>
<th>#2, male</th>
<th>#3, male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>5 years, 4 months</td>
<td>2 years, 10 months</td>
<td>4 years, 4 months</td>
</tr>
<tr>
<td>Sample</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Tooth</td>
<td>52</td>
<td>51</td>
<td>62</td>
</tr>
</tbody>
</table>

Live *H. pylori* or DNA found in:

| Root canal | - (+) | - (-) | + (+) | + (+) | - (-) | - (-) | - (-) | - (-) | - (-) |
| Plaque     | - (-) | - (-) | - (+) | - (+) | - (-) | - (+) | - (+) | - (+) | - (-) |

* Live *H. pylori* were identified by direct growth. The results of 16SrRNA gene PCR are given in parenthesis. Abbreviations: +, positive growth/PCR signal; -, no growth/PCR signal