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

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**Abstract**

*Background* Many polymerase chain reaction (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 still unclear whether *H. pylori* can indeed survive in the oral environment.

*Methods* Here we used electron microscopy, selective growth techniques, urease assays, 16S rRNA 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 by PCR in several plaque and root canal samples, bacterial colonies could only be grown from two root canals, but not from plaque. These colonies were unequivocally identified as *H. pylori* by microscopic, genetic, and biochemical approaches.

*Conclusions* Our findings show that root canals of endodontic-infected teeth may be a reservoir for live *H. pylori* that could serve as a potential source for transmission.

**Keywords** Deciduous teeth · Electron microscopy · Endodontics · *Helicobacter pylori* · Protein profiling · Root canal

**Introduction**

The International Agency for Research on Cancer has 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 is known to be transmitted in early childhood, the exact route of transmission is still unclear [3]. In a recent meta-analysis, a close relationship was found between *H. pylori* infection in the oral cavity and stomach; the authors concluded that *H. pylori* in the oral cavity was more difficult to eradicate than in the stomach, and may therefore be a source of gastric re-infections. However, the specific populated niche in the oral environment is unknown [4].

The majority of studies analysed specimens of dental plaque, saliva, or oral mucosa, and identified several *H. pylori* markers by various tests, such as the urea breath test.
test, rapid urease test, Campylobacter-like organism test, or polymerase chain reaction (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 or via the reflux of *H. pylori* or its DNA from the stomach to the mouth [6–8]; erroneous results can also arise from the misclassification of other urease-producing microorganisms. Thus, it is still unclear whether *H. pylori* can indeed survive in the oral environment. In this article we report two cases of 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 pediatric patients who received dental treatment under general anesthesia because of severe early childhood caries. Table 1 summarizes data about the patients’ age and gender, as well as the tooth numbers of 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 of 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 into 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 trimethoprim, 10 μg/ml nystatin, and 10 μg/ml colistin) for 7 days at 37°C, using the Campygen gas-generating system (all reagents from Oxoid/Fisher Scientific, Dublin, Ireland) [9, 10]. Single bacterial colonies were further analyzed. 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 agar was then acidified to pH 5 using 1 M HCl [11].

16S rRNA 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 and 2% glutaraldehyde in cacodylate buffer (0.1 mM cacodylate, 0.01 mM CaCl₂, 0.01 mM MgCl₂, 0.09 mM sucrose, pH 6.9). The 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 secondary electron (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% sodium dodecylsulfate-polyacrylamide (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 antiumease 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: DKIKVTPGSNKEY), FlaA (aa 93–106: KVKATQALSDDGQTT), VirB9 (aa 503–522: IKNYGELEVRKLPLVRDK), VirB10/CagY (repeat region: VSRARRNNEKEE), and Cag3/Cagδ (aa 32–45: IKATKETKET). Rabbit anti-CagM, anti-CagN, and anti-VacA antibodies were raised against all the recombinant proteins. These antibodies were affinity-purified and prepared according to standard protocols by Biogenes (Berlin, Germany). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit polyvalent sheep immunoglobulin was used as secondary antibody (DK-2600, DAKO, Glostrup, Denmark). Blots were developed with ECL Plus western blot reagents (GE Healthcare UK, Amersham, UK) as described [9].

**Results**

To investigate whether *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 have been 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 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 with tooth debris (Fig. 1a, yellow arrows). Several monopolar flagella were also observed, typical of *H. pylori* [10]. In addition, and as expected, coccoid bacteria of an unknown nature, which could also represent *H. pylori*, were observed (Fig. 1a, blue arrows). These morphological data suggested 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 \textit{H. pylori} urease \cite{11}. These experiments yielded functional urease enzymes allowing urea hydrolyzation in root canal samples to a high extent, similar to that in \textit{H. pylori} control strains, while retarded growth and no urea hydrolyzation was seen in \textit{DeltaureA} mutants or in any of the non-\textit{H. pylori} samples from dental plaque \cite{1b}. To unquestionably identify \textit{H. pylori}, we determined the 16S rRNA gene sequences from the two root canal isolates, as described \cite{10}. Both strains had completely identical sequences showing strong homology to that of several published \textit{H. pylori} strains \cite{2a}. To characterize our isolates further, we performed western blotting and confirmed the presence of several well-known \textit{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 \cite{2b}, arrows. In agreement with the observation of flagella by FESEM, we also found that our isolated root canal strains expressed the flagellin component FlaA \cite{4}. Moreover, the presence of certain adhesins (BabA, SabA, and OipA), \textit{cag} pathogenicity island encoded proteins (CagL, CagM, CagN, Cag3, VirB9, and VirB10), and other virulence factors (NapA, HtrA, Slt, and VacA) was also confirmed by western blotting, using specific antibodies (data not shown). Thus, our findings clearly indicate the successful isolation of live \textit{H. pylori} from the root canals of teeth.

\section*{Discussion}

\textit{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 unsuccessful \cite{5,12}. Here, live bacteria from two root canal samples were unequivocally identified as \textit{H. pylori}. To our knowledge, this is the first report of the recovery of viable \textit{H. pylori} from root canal samples, suggesting that this environment may be a reservoir for \textit{H. pylori} survival and growth that could serve as a potential source for the organism’s transmission. It is possible that these bacteria are of gastric origin, and that patients carrying \textit{H. pylori} in their dental root canals are also colonized by the same or different strains in the gastric mucosa. Colonization 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 \textit{H. pylori} which facilitates transmission among humans is a pressing question for future studies.

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\section*{Conflict of interest}

The authors declare that they have no conflict of interest.

\section*{References}