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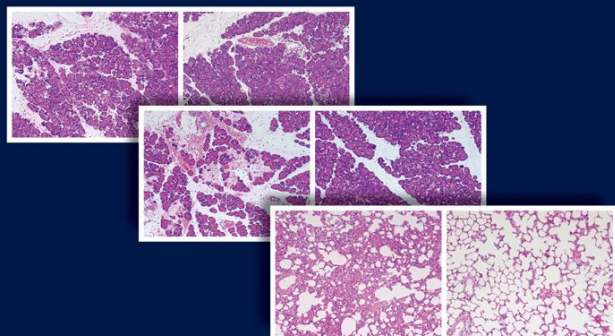
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Live *Helicobacter pylori* in the root canal of endodontic-infected deciduous teeth

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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

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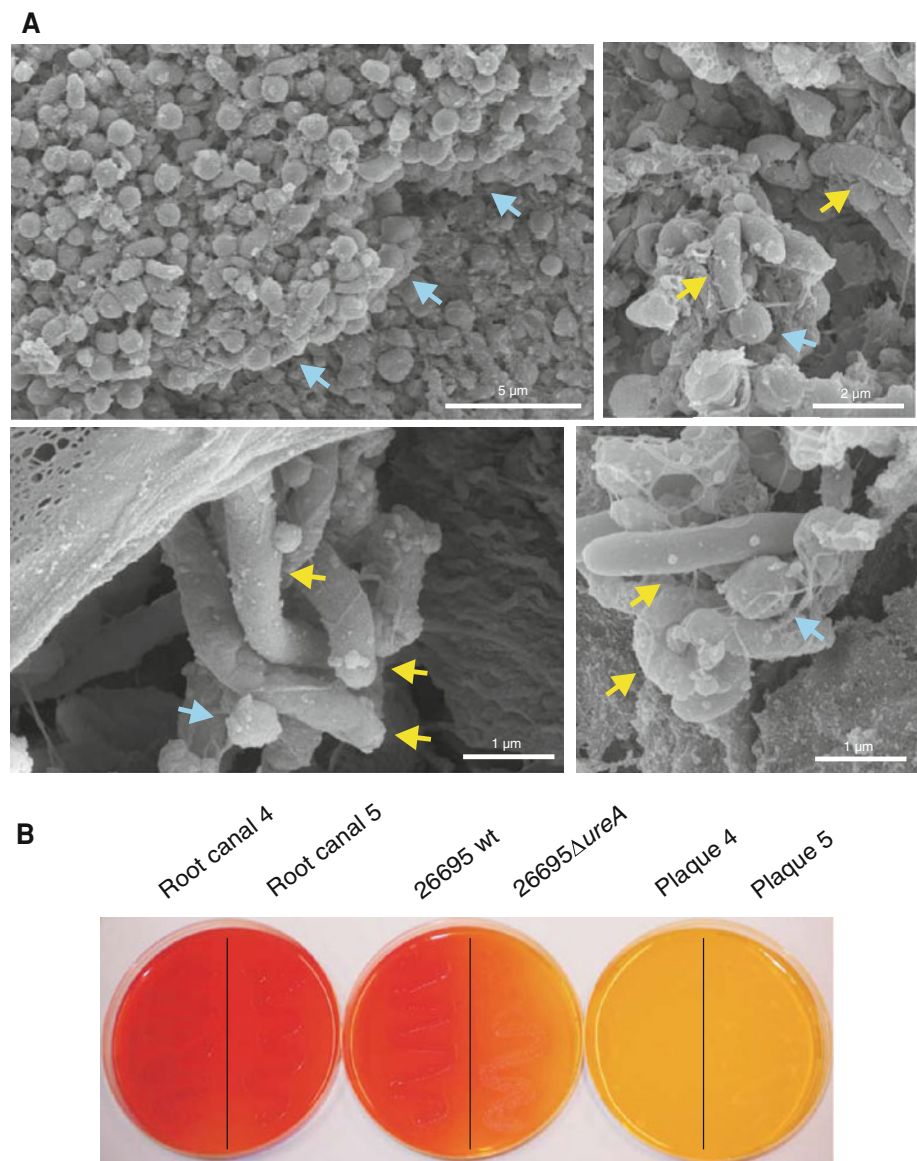
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Introduction

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

Fig. 1 Morphological analyses of two root canal samples (4 and 5) by field-emission scanning electron microscopy (FESEM) and urease tests. **a** FESEM revealed *Helicobacter pylori*-like spiral-shaped bacteria (yellow arrows) that were approximately 0.2 μm in diameter and varied in length from approximately 2 to 3 μm . Cocci bacteria 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 and 5 and strain 26695; the observed color change from orange to red indicated that bacterial colonies were producing functional urease and growing. *Right samples* 26695 Δ ureA and plaque samples 4 and 5. Color change did not occur in the right samples, indicating that functional urease was not being produced



with phenol red (100 $\mu\text{g}/\text{ml}$) and urea (600 $\mu\text{g}/\text{ml}$) as described [11]. The molten agar was then acidified to pH 5 using 1 M HCl [11].

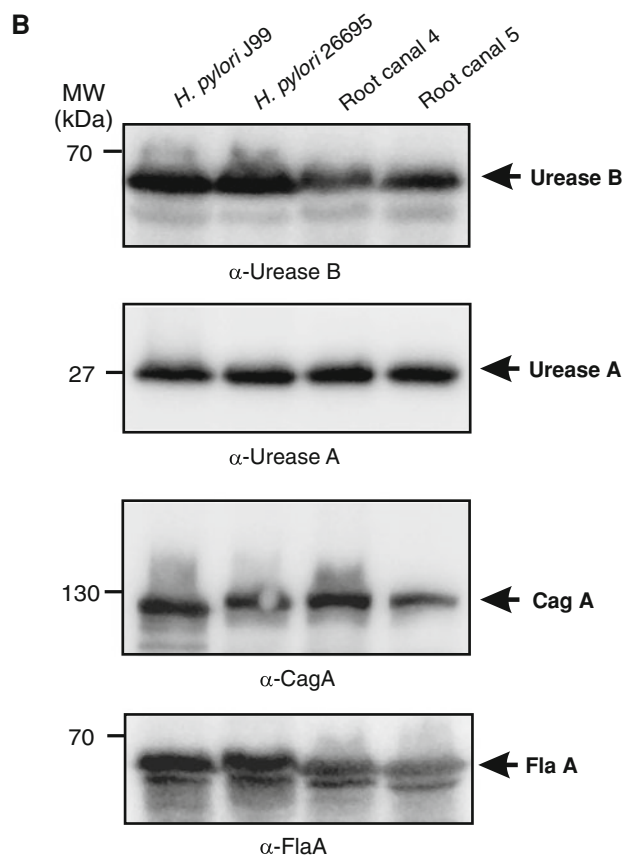
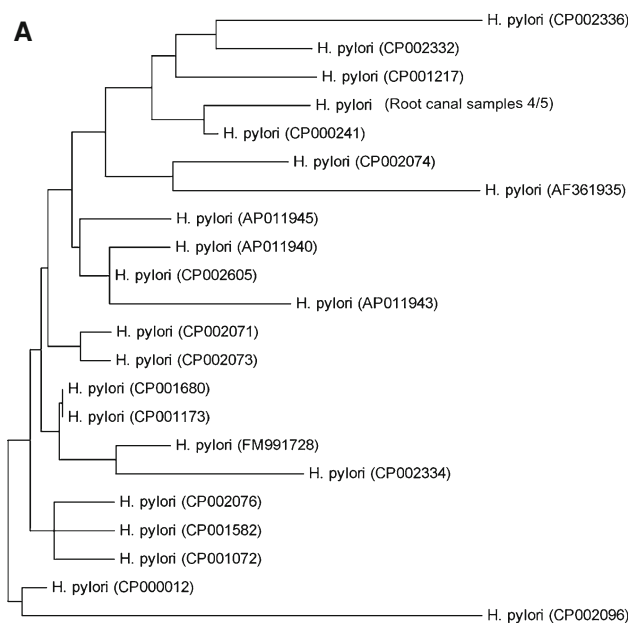
16S rRNA gene PCR and electron microscopy

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 CaCl_2 , 0.01 mM MgCl_2 , 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 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: CGGNANGQUESTSSTT), SabA (aa 172–186: CAMDQTTYDKMKKLA), OipA (aa 275–288: NYYSDDYGDKLDYK), NapA (aa 105–118: EFKELSNT

Fig. 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 urease A, urease B, CagA, and flagellin A (*FlaA*)

AEKEGD), Slt (aa 492–505: LRRWLESSKRFKEK), HtrA (aa 90–103: DKIKVTIPGSNKEY), FlaA (aa 93–106: KVK ATQAAQDGQTT), VirB9 (aa 503–522: IKNYGELERVIK KLPLVRDK), VirB10/CagY (repeat region: VSRARNEKE KKE), and Cag3/Cag δ (aa 32–45: IKATKETKETKKEA). 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). These candidate *H. pylori* were approximately 0.2 μ m in diameter and varied in length from 2 to 3 μ m. 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 *H. pylori* urease [11]. These experiments yielded functional urease enzymes allowing urea hydrolyzation in root canal samples to a high extent, similar to that in *H. pylori* control strains, while retarded growth and no urea hydrolyzation was seen in Δ ureA mutants or in any of the non-*H. pylori* samples from dental plaque (Fig. 1b). To unquestionably 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 (Fig. 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 (Fig. 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 (Fig. 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) was also confirmed by western blotting, using specific antibodies (data not shown). Thus, our findings clearly indicate the successful isolation of live *H. pylori* from the root canals 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 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 *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 *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 *H. pylori* which

facilitates transmission among humans is a pressing question for future studies.

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

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