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1 *Rapid Communication*

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3 Live *Helicobacter pylori* in the root canal of endodontic-infected 4 deciduous teeth

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30 **Key words:** deciduous teeth, electron microscopy, endodontics, *Helicobacter pylori*, protein
31 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.

1 **Introduction**

2

3 The International Agency for Research on Cancer characterized *Helicobacter pylori* as a type
4 I carcinogen [1] that is responsible for gastritis, gastro-duodenal ulcers, and gastric
5 malignancies in humans [2]. Although it is one of the most common infections in the world
6 and known to be transmitted in early childhood, the exact route of transmission is still unclear
7 [3]. In a recent meta-analysis, a close relationship between *H. pylori* infection in the oral
8 cavity and stomach was found; the authors concluded that *H. pylori* in the oral cavity is more
9 difficult to eradicate than in the stomach, and may therefore be a source of gastric re-
10 infections. However, a specific populated niche in the oral environment is unknown [4].

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

22

1 **Methods**

2

3 Patient selection and characteristics

4

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

13

14 DNA isolation and *H. pylori* growth

15

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

1 and urea (600 μg/ml) as described [11]. The molten was then acidified to pH 5 using 1M HCl
2 [11].
3
4 16SrRNA gene PCR and electron microscopy
5
6 For PCR amplification of the 16S rRNA gene in the genus *Helicobacter*, primers 5´-AGA
7 GTT TGA TYM TGG C-3´ and 5´-TAC GGY TAC CTT GTT ACG A-3´ were used, and
8 amplicons were sequenced as described [10]. For field-emission scanning electron
9 microscopy (FESEM), tooth samples were fixed in a sterile solution containing 5%
10 formaldehyde, 2% glutaraldehyde in cacodylate buffer (0.1 mM cacodylate, 0.01 mM $CaCl_2$,
11 0.01 mM $MgCl_2$, 0.09 mM sucrose, pH 6.9). Samples were subsequently covered with an
12 approximately 10 nm-thick gold film by sputter coating and examined in a field-emission
13 scanning electron microscope using an Everhart Thornley SE detector and in-lens detector in
14 a 50:50 ratio at an acceleration voltage of 5.0 kV as described [10].
15
16 Protein profiling and Western blotting
17
18 For protein profiling, pure plate-grown bacterial samples were run on 12% SDS-PAGE gels
19 and analyzed by Coomassie blue staining or Western blotting [9]. The following primary
20 antibodies were used: mouse monoclonal anti-CagA antibody (Austral Biologicals, San
21 Ramon, CA, USA), mouse polyclonal anti-urease antibodies [9], and polyclonal rabbit
22 antibodies recognizing a series of other *H. pylori* proteins. These antibodies were raised
23 against peptides corresponding to the following conserved amino-acid (aa) residues in *H.*
24 *pylori* strain 26695: BabA (aa 126–140: CGGNANGQESTSSTT), SabA (aa 172–186:
25 CAMDQTTYDKMKKLA), OipA (aa 275–288: NYYSDDYGDKLDYK), NapA (aa 105–
26 118: EFKELSNTAEKEGD), Slt (aa 492–505: LRRWLESSKRFKEK), HtrA (aa 90–

1 103:DKIKVTIPGSNKEY), FlaA (aa 93–106: KVKATQAAQDGQTT), VirB9 (aa 503–522:
2 IKNYGELERVIKKLPLVRDK), VirB10/CagY (repeat region: VSRARNEKEKKE), and
3 Cag3/Cagδ (aa 32–45: IKATKETKETKKEA). Rabbit anti-CagM, anti-CagN and anti-VacA
4 antibodies were raised against the entire recombinant proteins. These antibodies were affinity-
5 purified and prepared according to standard protocols by Biogenes GmbH (Berlin, Germany).
6 Horseradish peroxidase-conjugated anti-mouse or anti-rabbit polyvalent sheep
7 immunoglobulin was used as secondary antibody (DAKO Denmark A/S, DK-2600 Glostrup,
8 Denmark). Blots were developed with ECL Plus Western blot reagents (GE Healthcare, UK
9 limited Amersham Place, UK) as described [9].
10

1 **Results**

2

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

20 To exclude artifacts, bacteria were grown on selective acidified agar plates
21 supplemented with urea, the substrate of *H. pylori* urease [11]. These analyses yielded
22 functional urease enzymes allowing urea hydrolysation in root canal samples to high extent
23 similar to *H. pylori* control strains, while retarded growth and no urea hydrolysation was seen
24 in Δ*ureA* mutants or in all non-*H. pylori* samples from dental plaque (Figure 1B). To
25 unquestionable identify *H. pylori*, we determined the 16S rRNA gene sequences from the two
26 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.

1 **Discussion**

2 *H. pylori* can be cultured from human stomach biopsies, but attempts to identify other natural
3 reservoirs for these organisms or the routes by which they are transmitted to the stomach have
4 been yet unsuccessful [5, 12]. Here, live bacteria from two root canal samples were
5 unequivocally identified as *H. pylori*. To our knowledge, this is the first report of the recovery
6 of viable *H. pylori* from root canal samples, suggesting that this environment may be a
7 reservoir for survival and growth that could serve as a potential source for *H. pylori*
8 transmission. It is possible that these bacteria are of gastric origin, and that patients carrying
9 *H. pylori* in their dental root canals are also colonised by the same or different strains in the
10 gastric mucosa. Colonisation of the root canal may explain why eradication is often
11 unsuccessful as the antibiotic therapy used may not penetrate the root canal. Whether or not
12 this environment represents a reservoir for *H. pylori* which facilitates transmission among
13 humans is a pressing question for future studies.
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15
16
17 **Conflict of interest** The authors declare that they have no conflict of interest.
18

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1 **Figure Legends**

2

3 *Figure 1.* Morphological analyses of two root-canal samples (4 and 5) by FESEM and urease
4 tests. (A) FESEM revealed *H. pylori*-like spiral-shaped bacteria (yellow arrows) that were
5 approximately 0.2 µm in diameter and varied in length from approximately 2–3 µm. Coccoid
6 bacteria with 0.5–1 µm in diameter were also observed in large aggregates (blue arrows).
7 Representative pictures are shown from two preparations. (B) Selection of bacteria producing
8 functional urease on acidified agar supplemented with urea. Left samples: Root canal samples
9 4, 5 and strain 26695; the colour change from orange to red observed indicated that bacterial
10 colonies were producing functional urease and growing. Right samples: 26695Δ*ureA,* plaque
11 samples 4 and 5. Colour change did not occur on the right samples, indicating that functional
12 urease was not being produced.

13

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

19

1 *Table 1*. Sample Characteristics and *H. pylori* identification*
2

Patient	#1, male			#2, male			#3, male			
Age	5 years, 4 months			2 years, 10 months			4 years, 4 months			
Sample	1	2	3	4	5	6	7	8	9	10
Tooth	52	51	62	51	61	62	52	51	61	62
Live *H. pylori* or DNA found in:										
Root canal	- (-)	- (-)	- (-)	+ (+)	+ (+)	- (-)	- (-)	- (-)	- (-)	- (-)
Plaque	- (-)	- (-)	- (+)	- (+)	- (-)	- (+)	- (+)	- (-)	- (-)	- (-)

3 * Live *H. pylori* were identified by direct growth. The results of 16SrRNA gene PCR are
4 given in parenthesis. Abbreviations: +, positive growth/PCR signal; -, no growth/PCR signal
5