Detection of *Helicobacter pylori* in Dental Plaque by Reverse Transcription-Polymerase Chain Reaction

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To investigate whether the oral cavity is a potential reservoir and possible sanctuary for *Helicobacter pylori*, supragingival and subgingival plaques were analyzed by a *Helicobacter* genus-specific reverse transcriptase-polymerase chain reaction based on the sequence data of *H. pylori* 16S rRNA. The amplified 500-bp DNA fragment was identified by ethidium bromide staining after agarose gel electrophoresis and by Southern hybridization. Twenty-five dyspeptic patients were studied. Histologic examination of gastric biopsy specimens revealed that 18 had *H. pylori* gastritis and 7 did not. For seven of the 18 (38.8%) patients with proven *H. pylori* gastritis, *H. pylori* was also identified in their dental plaque. None of the patients without *H. pylori* gastritis had *H. pylori* in their dental plaque. The detection of *H. pylori* in dental plaque suggests that this *H. pylori* colonization is not restricted to the gastric mucosa and that this ecological niche may serve as a possible sanctuary which may be responsible for reinoculation of the stomach after topical anti-*H. pylori* therapies such as bismuth.

Helicobacter pylori is a fastidious, microaerophilic spiral gram-negative organism that is strongly associated with active and chronic gastritis as well as with peptic and duodenal ulcer diseases (11, 13). *H. pylori* infections are present worldwide. Although many aspects of the epidemiology of *H. pylori* infection are known, the mode(s) of transmission remains unclear. Recently, *H. pylori* has been found in association with dental plaque, suggesting that the oral environment may be one of the many potential pathways for transmission (17, 18, 20).

The microbiology of dental plaque is complex and includes numerous fragile and fastidious forms. Previous studies have identified the numerical dominance of gram-negative organisms in deep periodontal pockets and root canals (7, 22, 23). A number of asaccharolytic gram-negative rods including *Eikenella corrodens*, anaerobic *Vibrio* species, and *Campylobacter* species other than *Campylobacter sputorum* have been identified in dental plaque. Actively motile strains considered to be members of the genera *Wolinella* or *Campylobacter* have also been described (22, 23).

H. pylori infections are particularly difficult to eradicate, and it has been postulated that a sanctuary or sanctuaries which allow them to evade antimicrobial therapy must exist (2, 3, 10, 12). The advent of the polymerase chain reaction (PCR) has greatly simplified the development of sensitive and specific tests for detection of microorganisms because it requires only minute quantities of DNA or RNA. Several nucleic acid-based techniques have been developed to detect *H. pylori* (5, 14–16, 19, 24). The in vitro amplification of ribosomal 16S gene segments has a theoretical advantage, because the high copy number of rRNA per bacterial cell increases the target DNA copies (templates) by several thousand-fold (25). Thus, reverse transcription (RT) of RNA followed by PCR (RT-PCR) offers the theoretical advantages of high sensitivity and specificity when combined with

Southern hybridization and detection by using a PCR-amplified probe for detection of reaction products. We applied this RT-PCR protocol, recently developed in our laboratory (8), for the detection of *H. pylori* in dental plaque and to study whether the oral cavity may serve as a potential reservoir for *H. pylori*.

MATERIALS AND METHODS

Subjects. Dyspeptic patients referred for upper gastrointestinal endoscopy participated in the study. All patients gave informed written consent in accordance with the guidelines of the Committee on Human Investigation. The subjects' frequency of dental visits and their oral hygiene protocols were recorded. Subjects were excluded if they were edentulous or had a bleeding tendency (i.e., those who were taking anticoagulants), as were those with contraindications to endoscopy or those who required antibiotic prophylaxis for endoscopy or dental work (i.e., those with prosthetic heart valves).

Specimen collection. The patients' gingiva and plaque were assessed by using the gingival and plaque indices of Silness and Loe (21). The gingival index was as follows: 0, normal gingiva; 1, mild inflammation, slight change in color, slight edema, and no bleeding on probing; 2, moderate inflammation, redness, edema, and bleeding on probing; and 3, severe inflammation, marked redness and edema, ulceration, and tendency to spontaneous hemorrhage. The plaque index was as follows: 0, no plaque; 1, film of plaque, visible only on removal on probe or by disclosing with color indicator system; 2, moderate accumulation of deposits within the pockets or on the margins which can be seen with the naked eye; and 3, heavy accumulation of material filling the niche between the gingival margin and the tooth surface; the interdental region is filled with debris. Dental plaque was then removed from the tooth surfaces with a sterile periodontal curette. Both supra- and subgingival plaques were collected by an upward scrape against the tooth surface,

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immediately placed in sterile *H. pylori* brucella broth containing 20% glycerol storage medium (1), and were stored at -70°C until they were processed. Part of the plaque from 15 patients was placed on a clean glass slide to be used in the indirect immunofluorescence technique. Plaque specimens were always taken prior to the endoscopic procedure that was done to obtain a gastric mucosal biopsy specimen to exclude the possibility of contamination of the tooth surface with *H. pylori* during the withdrawal of the endoscope.

Sample processing. The sample was defrosted and transferred to a microcentrifuge with a disposable sterile plastic pipette. Insoluble material was harvested by centrifugation at 4°C for 10 min in a microcentrifuge. The supernatant was decanted and the precipitate was homogenized with a disposable pestle homogenizer (Kontes, Vineland, N.J.). The homogenate was resuspended and vortexed in 200 μl of lysis buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0], 10 mM NaCl, 1% Triton X-100). After incubation for 30 min at 100°C , the sample was extracted once by vortexing with an equal volume of phenol-chloroform-isoamyl alcohol (24:1; vol/vol) in Phase Lock Gel tubes (5'–3' Inc., Paoli, Pa.). After 15 min of centrifugation, the upper aqueous layer containing the nucleic acid was stored at -20°C until use.

RT-PCR conditions. Two oligonucleotide primers, designated HP1 (5'-TGGCAATCAGCGTCAGGTAATG-3') and HP2 (5'-GCTAAGAGATCAGCCTATGTCC-3'), were derived from *H. pylori* 16S rRNA as described previously (8). To investigate the sensitivity of *H. pylori* detection in dental plaque samples by the RT-PCR assay, the production of cDNA was performed as described previously (8). Briefly, the RT was carried out in a total volume of 30 μl containing PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.0], 2.5 mM MgCl_2 , 3% glycerol), the four deoxynucleotides (dATP, dCTP, dTTP, dGTP; 200 μM each), 180 ng of HP1 primer, 40 Units of RNasin (Promega Biotec, Madison, Wis.), 100 U of mouse mammary leukemia virus reverse transcriptase (GIBCO BRL), and a 10- μl volume of nucleic acid extracts. After 60 min of incubation at 37°C , a total volume of 70 μl containing PCR buffer, 180 ng of HP2 primer, and 2.5 U of AmpliTaq polymerase was added. Each reaction was overlaid with 50 μl of light mineral oil to prevent evaporation. The reaction mixtures were subjected to an initial denaturation step at 94°C for 5 min; this was followed by 39 cycles of amplification performed in a programmable thermal controller (MJ Research, Watertown, Pa.) as follows: 1 min of denaturation at 94°C , 1 min of annealing at 55°C , and 3 min of primer extension at 72°C . After the 39th cycle, the extension step was continued for another 10 min at 72°C and the product was stored at 4°C until use. Twenty microliters of each amplified sample was analyzed by 1.5% agarose gel electrophoresis with 0.5 μg of ethidium bromide per ml used as a stain. The presence of PCR DNA products migrating at approximately 500 bp was visualized, and the products were photographed under UV light.

Southern hybridization analysis. Amplified target sequences from the reference strain (*H. pylori* RD26) were used as probes in Southern blot hybridizations. The DNA probes were prepared by the hexanucleotide priming technique with digoxigenin-11-dUTP by using the Genius labeling kit and following the manufacturer's instructions (Boehringer Mannheim, Indianapolis, Ind.). The amplified PCR DNA fragments were transferred onto Hybond N^+ nylon membranes (Amersham, Arlington Heights, Ill.) by the alkaline blotting procedure, and the hybridization procedure was performed as described previously (8).

Indirect immunofluorescence. Dental plaque samples were

examined for *H. pylori* by indirect immunofluorescence microscopy with genus-specific E7C11 monoclonal antibody reagents (9). Briefly, the plaque samples smeared on 15 slides were covered with undiluted supernatant of monoclonal antibody E7C11 for 45 min at 37°C in a moist chamber. The slides were rinsed gently with phosphate-buffered saline (PBS; 0.01 M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.15 M NaCl [pH 7.0]) and were stored for 10 min in the same buffer. Fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G antibodies (Fisher Biotec, Pittsburgh, Pa.) at 1:200 dilutions in PBS were added over the smears and the smears were incubated as described above. After gentle washing in PBS, slides were examined by UV light microscopy. *H. pylori*-positive controls as well as negative controls (without primary antibody) were included.

Histologic confirmation of *H. pylori* status. Gastric mucosal biopsy specimens were taken with jumbo forceps (13-K with spike removed; Olympus, Lake Success, N.Y.). Biopsy specimens, which were fixed in 10% buffered formalin, were processed, oriented on edge, embedded in paraffin, and cut in sequential 4- μm sections. Virtually all specimens included surface epithelium and muscularis mucosae. From each specimen, one slide (usually with 8 to 12 sections) was stained with hematoxylin and eosin, one was stained with periodic acid Schiff, one was stained with a Genta stain (a combination of a Steiner stain, hematoxylin and eosin and Alcian Blue at pH 2.5) for the visualization of *H. pylori*, and one was stained with the modified Rubin's stain (a combination of hematoxylin and eosin, Alcian Blue at pH 2.5, and Metanil Yellow). The presence and density of *H. pylori* and the degree of inflammatory responses of the gastric mucosae were assessed and graded semiquantitatively, and the results were scored as *H. pylori* infected or uninfected on the basis of the presence of the organism and the presence of typical acute-on-chronic gastritis.

RESULTS

Dental plaque and gastric mucosal biopsy specimens were obtained from 25 men ranging in age 24 to 72 years; 16 were black and 9 were white. Clinical diagnoses included duodenal ulcer, gastric ulcer, and nonulcer dyspepsia (including gastroesophageal reflux). Histological examination of the gastric mucosal biopsy specimens revealed that 18 patients had *H. pylori* gastritis and that 7 were not infected.

RT-PCR assay of dental plaque samples. Our RT-PCR assay was previously proved to be highly sensitive for *H. pylori* (8); the expected amplified product was detected from as few as one to two organisms per reaction on agarose gels. In preliminary experiments, in which normal dental plaque samples were spiked with a known number of *H. pylori* RD26 organisms, we were able to detect the expected amplified 500-bp fragment from as few as 10 organisms on agarose gels, and this was increased about 10-fold on Southern blots (data not shown). Eighteen of the 25 patients had gastric *H. pylori* infection proven by histology, and 7 of these (38.8%) had *H. pylori* infection identified by RT-PCR in dental plaque (Table 1). All results were true positives on the basis of histologic identification of *H. pylori*-associated gastritis; there were no false-positives results because the RT-PCR results for all seven patients without *H. pylori* gastritis were negative. As shown in Fig. 1, five dental plaque samples were positive for *H. pylori* by gel electrophoresis and two additional samples were identified as

TABLE 1. Relation between dental hygiene and recovery of *H. pylori*

Dental hygiene parameters	No.		
	Patients	Infected patients ^a	Patients with PCR-positive plaque
Dental visit (no./yr)			
<1	2	1	0
1-5	12	10	4
>5	11	7	3
Oral hygiene (no. of times)			
Daily (1 or 2)	19	15	5
Weekly (≤ 4)	3	2	1
More than weekly	3	1	1

^a *H. pylori* gastritis by histology.

positive by Southern hybridization with the *H. pylori* PCR probe.

The effect of a possible inhibitory factor(s) in RT-PCR-negative dental plaque specimens was tested in specimens from 10 of the 11 patients with *H. pylori*-associated gastritis by adding *H. pylori* nucleic acid equivalent to 180 organisms

TABLE 2. Relationship between gingival and plaque indices and presence of *H. pylori* determined by RT-PCR

Index	No.		
	Patients	Infected patients ^a	Patients with PCR-positive plaque
Gingival			
0	0		
1	5	4	1
2	15	11	5
3	5	3	1
Plaque			
0	0		
1	2	2	0
2	10	6	4
3	13	10	3

^a *H. pylori* gastritis by histology.

per reaction. All the spiked reactions showed the expected product. The intensities of these products varied among these samples, suggesting the presence of possible inhibitory elements in some of these dental plaque specimens.

H. pylori was identified from the subgingiva of both the anterior and the posterior regions. Positive PCR results were equally distributed in both supra- and subgingival plaques and from the anterior-posterior region (i.e., the ratio was 4:3). The frequency of *H. pylori* recovery did not increase with increasing gingival or plaque index. *H. pylori* was not detected in the two patients with plaque indices of 1 and was found in 28% of those with plaque indices of 2 and 3 (Table 2).

Six of seven patients whose dental plaques were positive by RT-PCR reported symptoms of reflux, gas production, hematemesis, and vomiting ranging from once to six times a day for weeks or months prior to being studied.

Indirect immunofluorescence microscopy. Fifteen plaque samples were tested for *H. pylori* organisms by indirect immunofluorescence by using monoclonal antibodies; two plaque samples (13.3%) were positive. These two samples were also positive by RT-PCR. In these two samples, the areas of stained bacteria were localized in the largest conglomeration of plaque smears and both were obtained from the posterior region.

DISCUSSION

Despite the potential problems with culture of *H. pylori* from dental plaque samples, there have been two reports of its isolation from dental plaque (17, 18). The frequency of isolation has varied; Krajden et al. (17) in Canada reported that for only 1 of 29 patients (3.4%) with *H. pylori* gastritis was *H. pylori* recoverable from their dental plaques. In contrast, in India *H. pylori* was present in 100% of 40 volunteers (18). By PCR, *H. pylori* has also been identified in the saliva of 9 of 19 patients (47%) with *H. pylori* gastritis (14). Using the RT-PCR technique, we identified *H. pylori* in dental plaque samples from 38% of patients with *H. pylori* gastritis. We also found that the frequency of dental visits, oral hygiene, plaque deposits and gingival inflammation index, and the location of the plaque (supragingival versus subgingival and anterior versus posterior) did not predict the presence of *H. pylori* in dental plaques. These studies taken together strongly suggest that the oral cavity in general, and

FIG. 1. Representative RT-PCR amplification applied to dental plaque specimens. (A) Agarose gel electrophoresis; (B) corresponding Southern blot hybridization. Crude extracts from 10 dental plaque specimens (lanes 1 to 10) obtained from patients with *H. pylori*-associated chronic gastritis were used for amplification. Lane A, 180 *H. pylori* RD26 organisms per reaction; lane B, lysis buffer only (negative control); lane M, *Hind*III-digested bacteriophage lambda DNA.

that the dental plaque in particular, is a site of *H. pylori* colonization and may be an important reservoir of *H. pylori*. This hypothesis is supported by the recent report of Shames and coworkers (20), who used restriction endonuclease analysis of DNA and who reported that the same strain of *H. pylori* was present in the stomach and dental plaque. Finally, the fact that we took two samples from each individual but usually recovered *H. pylori* from only one sample suggests that *H. pylori* may not be uniformly distributed in the mouth and that more extensive sampling may lead to an even higher percentage of coinfection of the stomach and the oral cavity, as was found in India (18). It appears unlikely that the presence of *H. pylori* was missed because of technical difficulties because *H. pylori* is recoverable from infected tissue samples stored frozen in this medium for at least 2 years (unpublished data). There was no loss of RNA activity in our nucleic acid preparations since the amplified bands from positive samples gave the same intensities when they were used after three to four freeze-thaw (on ice) cycles, and hence, the presence of significant inhibitors in these samples was excluded. However, improving the quantity and quality of DNA templates by the addition of an RNase inhibitor(s) into the storage medium and by using different nucleic acid extraction and purification methods may increase the RNA recovery and reduce or eliminate some of the inhibitory effects in those RT-PCR-negative, *H. pylori* histology-positive samples.

The mechanism by which *H. pylori* reaches the oral cavity is unknown. It is possible that the occasional reflux from the gastric reservoir allows colonization of the oral cavity. It is also possible that the reverse is true. Desai et al. (6) reported that *H. pylori* was present in the dental plaques of patients when it was not present in their stomachs (6). The data from that study are difficult to interpret because the investigators used a rapid urease test as the detection method and the oral cavity contains many urease-containing bacteria other than *H. pylori* which may have caused false-positive results. Topical monotherapy (e.g., swallowing a tablet of bismuth subcitrate) for *H. pylori* infection has generally been unsuccessful (4). The therapeutic results suggest that recrudescence of infection after cessation of therapy may occur owing to recolonization of the stomach from the *H. pylori* present in dental plaque, because the latter would be unaffected by such treatment. Thus, a small number of organisms probably survive a treatment course only to multiply and recolonize when the treatment regimen is finished, rendering the patient liable to ulcer relapse. Studies that use sensitive and specific methods of *H. pylori* detection are needed to address whether *H. pylori* in dental plaque is possibly responsible for the failure to eradicate *H. pylori* and whether a search for *H. pylori* in the oral cavity would be useful for monitoring the efficacy of therapy. Furthermore, the ability to detect *H. pylori* in dental samples offers the potential for a noninvasive test of infection and would lead to considerable support to the notion that spread by the oral route is the principal mode of transmission of *H. pylori*.

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