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Identification by PCR of *Helicobacter pylori* in subgingival plaque of adult periodontitis patients

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The PCR was used to detect the presence of *Helicobacter pylori* in subgingival plaque samples from patients with adult periodontitis. Primers based upon the 16S ribosomal RNA (rRNA) gene sequence of *H. pylori* were used in a single round of PCR to amplify a 295-bp DNA fragment and the identity of the amplified products was confirmed by Southern blot hybridisation to a digoxigenin-labelled *H. pylori* probe. Further confirmation of product identity was obtained by DNA sequencing of a proportion of the amplified products. The assay was demonstrated to be specific for *H. pylori* with a lower limit of detection of 100 fg of bacterial genomic DNA. In all, 73 samples from 29 patients were analysed, of which 24 (33%) were *H. pylori*-positive by PCR; the proportion of patients carrying *H. pylori* in at least one sampled site was 38% (11 of 29). This is the first study to demonstrate the presence of *H. pylori* in the subgingival plaque of patients with adult periodontitis and indicates that, in this patient group at least, subgingival plaque may be a reservoir for *H. pylori* infection.

Introduction

*Helicobacter pylori* is a gram-negative, micro-aerophilic bacterium which is recognised as being an aetiological agent of chronic active gastritis and peptic ulcer disease [1–3], and has also been associated with gastric cancer [4, 5]. In the Western world, average *H. pylori* infection rates in healthy adults are estimated to be between 10 and 40%, whereas patients with gastritis or gastric and duodenal ulceration have infection rates of 80–100% [6]. The prevalence of *H. pylori* infection has been shown to increase with age, with an estimated 50–60% of people over 55 years old being infected, compared with only 10–20% of young adults [7, 8]. In developing countries, where sanitation is poor, up to 50% of children are infected by 5 years of age [9, 10].

The mode of transmission of *H. pylori* is poorly understood, although it appears that oral–oral and faecal–oral routes are possible [11]. Evidence exists for possible oral transmission from person-to-person [12, 13] and faecal–oral transmission is thought to be particularly prevalent in developing countries, although the organism is rarely isolated from faeces [13]. Transmission of *H. pylori* in gastric juice as a result of epidemic childhood vomiting has also been proposed [14]. The natural reservoir for *H. pylori* is unknown, although the oral cavity has been the focus of much attention in this respect. However, controversy surrounds the hypothesis that the oral cavity may be a permanent reservoir for *H. pylori*. The presence of *H. pylori* in dental plaque of patients with and without stomach disorders has been investigated by culture and PCR methods. Most studies have failed to isolate *H. pylori* by culture from dental plaque of patients undergoing endoscopy [15–19]. PCR analysis of dental plaque from endoscopy patients has yielded more conflicting data, with *H. pylori* found frequently in some studies [15, 20, 21], but absent or found at very low frequency in other studies [16, 22]. In the dental plaque of healthy subjects, culture methods have detected *H. pylori* at high frequency in a single study [23], and PCR has detected the organism at high frequency in only one study [15]. In symptomatic individuals, PCR has detected *H. pylori* in saliva [24–26], whereas culture methods have very rarely isolated *H. pylori* from saliva [27]. Clearly, the prevalence of *H. pylori* will vary amongst different population groups and this may account partly for the conflicting data presented in the literature on the presence of this organism in the oral cavity.

Previous studies have examined mostly either supra-gingival plaque alone or supragingival mixed with subgingival plaque from the gingival crevice of periodontally healthy individuals for the presence of
H. pylori. As Glasgow has a rate of H. pylori infection (70%) that is significantly higher than the UK average, this study aimed to investigate the possible presence of H. pylori in subgingival plaque of adult periodontitis patients attending Glasgow Dental Hospital for treatment. Only one previous study has reported the use of PCR to attempt detection of H. pylori in the subgingival plaque of adult periodontitis patients, but the organism was not found in any of the 336 samples analysed [28].

Materials and methods

Bacterial culture and genomic DNA purification

H. pylori (ATCC 43504) was inoculated on to tryptic soy agar plates containing horse blood 7% v/v, triphenyl tetrazolium chloride 0.1% w/v and vancomycin 3 mg/ml and incubated at 37°C in jars containing an atmosphere with increased CO₂ for 3–5 days. Bacterial growth from these plates was scraped off and genomic DNA was extracted with the Puregene DNA Purification Kit for gram-negative bacteria (Flowgen Instruments, Lichfield).

Sample details

Subjects were patients with chronic adult periodontitis who were newly referred to Glasgow Dental Hospital. Patients had at least three periodontal pockets with a minimum depth of 5 mm and bleeding on probing, had not received antibiotic therapy during the preceding 6 months and demonstrated no symptoms of gastritis or peptic ulcer disease. Two-to-four samples were analysed from 29 patients and of the 73 samples analysed, 40 were from 15 males (age range 36–54 years, mean age 45.9 years) and 33 were from 14 females (age range 36–60 years, mean age 44.1 years). In the male patient group, mean pocket depth of samples analysed was 5.8 mm (pocket depth range 5–9 mm) and for the female patient group mean pocket depth was 6.1 mm (pocket depth range 5–11 mm).

Collection of subgingival plaque samples

Subgingival plaque samples were collected with a single stroke of a separate sterile curette for each sample to prevent cross-contamination and placed into sterile tubes containing 0.5 ml of freshly prepared Fastidious Anaerobe Broth (Bioconnections, Leeds). Samples were vortex mixed for 30 s and lysates were prepared for use in PCR by addition of 90 µl of subgingival plaque to 10 µl of 10 × lysis buffer (100 mM Tris-HCl, pH 8.0; 10 mM EDTA; Triton X-100 10%) and boiling for 5 min. A 5-µl sample of lysate was used in each PCR reaction and the remainder of the lysates were stored at −70°C.

PCR primers

The primers selected for PCR targeted the 16S ribosomal RNA (rRNA) gene of H. pylori. A previously published study described the use of primer pair JW21/JW22 for the specific detection of H. pylori [29]. The present study used primer JW22 (5'-CGTTAGCTGATTACTGGAG-3'; nucleotides 830–810) but replaced JW21 with primer JW23 (5'-GAGC-GCCTAGCCGGATAGTGC-3'; nucleotides 536–556), which lies 156 nucleotides upstream of the JW21 primer sequence in the 16S rRNA gene. Primer JW23 shows more sequence diversity than primer JW21 at its 3' end with the corresponding region of the 16S rRNA genes of other closely related members of the Helicobacter genus and can thus be expected to be no less specific than primer JW21. The expected size of the amplification product obtained with primers JW22/JW23 was 295 bp.

PCR amplification

PCR amplification was performed in a reaction volume of 50 µl consisting of 5 µl of subgingival plaque lysate or 1 µl (100 ng) of H. pylori genomic DNA and either 45 or 49 µl of reaction mixture containing 1 × PCR buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM MgCl₂, Triton X-100 0.1%), 1.0 unit of Taq DNA polymerase (Pharmacia Biotech, St Albans), 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP) and each primer at a concentration of 0.2 µM. Primers were separated from other reaction components by a layer of wax ('hot start' PCR), preventing the reaction from starting until the wax melted upon commencement of thermal cycling and thus improving the specificity and yield of reaction products. PCR amplification was performed in an OmniGene thermal cycler (Hybaid, Teddington). After an initial denaturation step of 94°C for 5 min, there were 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. Reaction products were either analysed immediately or stored at −20°C until required.

PCR quality control

To avoid contamination, stringent procedures were employed when performing PCR. Separate rooms were used for sample preparation, setting up of PCR reactions and post-PCR analysis of reaction products. Filter tips were used at all stages when setting up reactions and positive displacement tips were used for adding sample to the reaction mixtures. Positive and negative controls were included for each set of samples analysed. The positive control used was a standard reaction mixture containing 100 ng of H. pylori genomic DNA instead of sample; the negative control contained sterile water instead of sample. A negative control was included for every three samples analysed.
Agarose gel electrophoresis

A 10-μl volume of each reaction product was added to 2 μl of gel loading dye (bromophenol blue 0.25% w/v, glycerol 50% v/v, 100 mM EDTA, pH 8.0), electrophoresed on an agarose 2% gel containing ethidium bromide 0.5 μg/ml and visualised on an ultraviolet (UV) transilluminator. A 100-bp DNA ladder (Pharmacia Biotech) was used as a size marker.

Southern blot hybridisation

Reaction products were electrophoresed on agarose 2% gels as described above and transferred to positively charged nylon membranes (Boehringer Mannheim, Lewes). Briefly, gels were prepared for transfer by washing in 250 ml of denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 2 × 20 min, followed by washing in 250 ml of neutralisation solution (0.5 M Tris-HCl, pH 7.4, 3.0 M NaCl) for 2 × 20 min. DNA was transferred to membranes by means of a capillary blotting unit (Anachem, Luton) with 20 X SSC (3.0 M NaCl, 0.3 M tri-sodium citrate, pH 7.0) as a transfer buffer. After overnight transfer, membranes were rinsed in 2 X SSC and DNA was immobilised to membranes by exposure to an optimal dose of UV energy in a cross-linker (UVC-508; Anachem).

Membranes were hybridised overnight at 68°C with the 295-bp H. pylori 16S rRNA PCR product labelled with digoxigenin (DNA Labelling and Detection Kit; Boehringer Mannheim) at 5 ng/ml in standard hybridisation buffer 5 × SSC, blocking reagent 1% w/v, N-laurylsarcosine 0.1% w/v, SDS 0.02% w/v). Membranes were rinsed at room temperature in 2 X SSC/SDS 0.1% for 2 × 5 min and then washed under conditions of high stringency at 68°C in 0.1 × SSC/SDS 0.1% for 2 × 20 min. Immunological detection of hybridised products was done according to the manufacturer’s instructions with an anti-digoxigenin antibody conjugated to alkaline phosphatase, followed by colorimetric detection with 4-nitroblue tetrazolium by exposure to an optimal dose of UV energy in a cross-linker (UVC-508; Anachem).

DNA sequencing

Selected PCR products were cloned into pCR2.1-TOPO cloning vector with the TOPO TA Cloning Kit (Invitrogen BV, NV Leek, The Netherlands) according to the manufacturer’s instructions. Plasmid DNA from recombinant clones was purified with the Wizard Plus SV Minipreps DNA Purification System (Promega Corporation, Southampton). Sequencing reactions were performed with Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Little Chalfont) and IRD800-labelled M13 universal (-21) primer. Sequence data were collected and analysed by an LICOR 4200S automated sequencing system (MWG-Biotech, Milton Keynes).

Results

Specificity of the H. pylori PCR assay

The H. pylori PCR assay was performed with 100 ng of genomic DNA from each of the oral species Porphyromonas gingivalis (ATCC 33277), Prevotella intermedia (ATCC 25611), Pr. nigrescens (ATCC 33563), Actinobacillus actinomycetemcomitans (ATCC 33384), Haemophilus aphrophilus (ATCC 33389), Ha. paraphrophilus (ATCC 29241), Bacteroides forsythus (ATCC 43037), Fusobacterium nucleatum (ATCC 25586), Eikenella corrodens (ATCC 23834), Streptococcus mutans (ATCC 25175), S. mitis (ATCC 49546) and Candida albicans (NCTC 3153), as well as genomic DNA from Escherichia coli (ATCC 11775). Genomic DNA from the closely related species H. cinaedi (ATCC 35683), H. mustelae (ATCC 43772), H. fennelliae (ATCC 35684), Campylobacter jejuni (ATCC 33560) and C. coli (ATCC 33559) were also tested. No DNA from any of these species was detectable by PCR (results not shown), thus confirming the specificity of the PCR assay for H. pylori.

Sensitivity of the H. pylori PCR assay

The lower detection limit after 40 cycles of DNA amplification with primers JW22/JW23 was 100 fg of H. pylori DNA, which is equivalent to c. 100 bacterial cells (results not shown).

Detection of H. pylori in subgingival plaque samples by PCR

The PCR assay specific for H. pylori was applied to subgingival plaque samples from periodontal pockets at least 5 mm in depth from 29 patients with adult periodontitis. Two-to-four subgingival plaque samples from different periodontal pockets were analysed for each patient and the presence of H. pylori was indicated by the amplification of a 295-bp product. H. pylori DNA was detected in 24 (33%) of the 73 samples analysed (14 male, 10 female); 11 (38%) of the 29 patients (6 male, 5 female) harboured H. pylori in at least one analysed site. H. pylori-positive patients harboured the organism in all the subgingival plaque samples analysed. Fig. 1 shows representative results from agarose gel electrophoresis of PCR products from subgingival plaque samples; amplified products were always a single band of the expected size (295 bp) and no non-specific reaction products were seen on any occasion.

Confirmation of product specificity

The specificity of amplified products was confirmed by Southern blot hybridisation to a digoxigenin-labelled H. pylori 16S rRNA probe (Fig. 2). All samples positive by agarose gel electrophoresis hybridised to the probe, confirming the successful amplification of
Fig. 1. Agarose 2% gel electrophoresis of selected products from subgingival plaque samples following PCR with the *H. pylori*-specific primer pair JW22/JW23. Lane M, 100-bp DNA ladder; 1–17, subgingival plaque samples; 18, negative control; 19, positive control. PCR positivity is indicated by the presence of a 295-bp product.

Fig. 2. Corresponding Southern blot hybridisation of the PCR products shown in Fig. 1. The PCR products were hybridised to the 295-bp *H. pylori* positive control 16S rRNA PCR product labelled with digoxigenin.

*H. pylori* DNA. No samples that were negative by agarose gel electrophoresis hybridised to the probe. Additional confirmation of product specificity was obtained by DNA sequencing of PCR products. Five PCR products were cloned into pCR2.1-TOPO cloning vector and four recombinant clones derived from each cloned product were sequenced. The DNA sequence data demonstrated that all 20 recombinant clones sequenced originated from *H. pylori* DNA, thus further confirming the specificity of the PCR assay.

Discussion

The purpose of this study was to examine subgingival plaque from adult periodontitis patients for the
presence of *H. pylori* with an *H. pylori*-specific PCR assay as described previously [29], but with some modifications. Although the primers used in the earlier study were shown to be specific for *H. pylori* at an annealing temperature of 55°C, in the present study the upstream primer was replaced with another that showed even more specificity at its 5' end and the annealing temperature was increased from 55°C to 60°C to further minimise non-specific annealing. The majority of published studies have examined dental plaque from subjects without periodontitis, i.e., supragingival plaque or subgingival plaque from the gingival crevice. A single study has examined subgingival plaque from adult periodontitis patients for *H. pylori* by PCR [28]. In the latter study, in the USA, none of 336 subgingival plaque samples analysed was positive for *H. pylori*. These results are clearly discrepant with the findings of the present study, in which *H. pylori* was found in the subgingival plaque of approximately one-third of the adult periodontitis patients examined. This difference may be attributable to the different population groups examined and also the methods employed for plaque sampling. The authors of the American study conceded that their paper point sampling method may collect less bacteria than the curette sampling method which was used in the present study. This less effective sampling method will undoubtedly affect the detection of *H. pylori* cells in the periodontal pocket if only low numbers of *H. pylori* cells are present.

The possible role of the oral cavity as a reservoir for *H. pylori* is a highly controversial issue. While some studies have detected *H. pylori* frequently at sites, such as plaque [15, 20, 21] and saliva [24–26], in the oral cavity of endoscopy patients by PCR, other studies have either failed to detect the organism or found it at very low frequency [16, 22]. Clearly, such conflicting data can be attributed in part to differences in the populations examined, as *H. pylori* is generally found at a higher prevalence in the populations of developing countries. It is not known whether *H. pylori* can colonise the oral cavity or is transient as a result of gastric reflux. Genetic typing studies on isolates obtained from both the oral cavity and stomach of patients have shown that strains from these two sites tend to be identical, although different strains are harboured by different individuals [19, 30]. The identification of identical strains of *H. pylori* in stomach and oral samples of individuals can be interpreted in two ways. Firstly, such observations could lend credence to the theory that *H. pylori* may be only a transient member of the oral cavity as it may be present in the oral cavity as a direct result of gastric reflux. Alternatively it can be speculated that oral sites such as dental plaque may be a reservoir for gastric re-infection by *H. pylori*. PCR has demonstrated the presence of *H. pylori* in the saliva of patients whose gastric biopsies were negative for *H. pylori* and lack of correlation between gastric symptoms and the presence of *H. pylori* in the oral cavity was also observed [25]. These observations led the authors to suggest that oral colonisation by *H. pylori* may precede gastric infection and that saliva may also be a reservoir for gastric re-infection by *H. pylori*. Claims that *H. pylori* is a permanent member of the oral cavity that can cause gastric re-infection, and inevitably person-to-person transmission, can be substantiated only by recovery of viable *H. pylori* from the oral cavity, which has rarely been accomplished. Reasons for the general inability to culture *H. pylori* from the oral cavity are difficult to assign with any certainty, but could include the presence of the organism in a coccoid form that is non-culturable in *vitro* [31]. Experimental evidence has been presented which suggests that the coccoid form of *H. pylori* may be viable [32, 33]. It has been postulated that *H. pylori* may exist in the environment in a non-culturable coccoid form that can revert to a replicative form *in vivo* following ingestion by a mammalian host [34], but no conclusive evidence exists to support this hypothesis. If this hypothesis could be proved it would strengthen the notion of water and food as important reservoirs for *H. pylori*.

Previous attempts to detect *H. pylori* in the oral cavity have focused primarily on dental plaque and, more rarely, saliva as target specimens. Recent studies have investigated other oral sites for the potential presence of *H. pylori*. *H. pylori* was not detected in any of 20 salivary lympho-epithelial lesions analysed by nested PCR [35]. In another study, nested PCR was used in the attempted detection of *H. pylori* in specimens from several sites in the oral cavity, i.e., lingual, buccal and labial mucosa, hard and soft palate, oral vestibule and gingiva [36]. Of the 161 patients examined, only 21 (13%) were PCR-positive for *H. pylori* and no site was identified as preferentially harbouring the organism; furthermore, no association was found between the presence of *H. pylori* and ulcerated (recurrent aphthous ulcers, oral erosive lichen planus, lingual ulcers) versus non-ulcerated lesions. PCR has also been used to identify *H. pylori* in the cheeks of dyspeptic patients [37]. *H. pylori* has been detected in six (21%) of 29 oral mucosal ulcers examined by in-situ hybridisation [38].

The majority of published studies that have identified *H. pylori* in oral samples have used two rounds of PCR for increased sensitivity. It is important to note that, in the present study, *H. pylori* DNA was amplified from subgingival plaque samples with a single round of PCR, with a lower detection limit of 100 fg of *H. pylori* DNA (c. 100 bacterial cells). Whereas two rounds of PCR can generally detect between one and 10 bacterial cells, the clinical significance of such small numbers of bacteria in clinical samples remains questionable. Although the PCR assay used in the present study is only semi-quantitative, many of the PCR-positive subgingival plaque samples analysed gave a signal intensity of a
magnitude where it could be expected that \textit{H. pylori} is present in numbers above the lower detection limit of culture methods. Future studies will aim to establish whether \textit{H. pylori} in PCR-positive subgingival plaque samples from adult periodontitis patients can indeed be isolated by culture or if organisms are present in a non-culturable form.

References