Identification of *Helicobacter pylori* DNA in the mouths and stomachs of patients with gastritis using PCR

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Abstract

Aims—To determine the prevalence of *Helicobacter pylori* colonisation in the mouths of patients with *H pylori* gastritis.

Methods—A nested polymerase chain reaction test for the 16S ribosomal RNA gene of *H pylori* was used on saliva, dental plaque, gastric juice and gastric biopsy specimens from patients attending a dyspepsia clinic.

Results—Thirteen patients had histologically confirmed Helicobacter associated gastritis. Twelve of these had positive gastric aspirates by PCR. Five had at least one positive oral specimen. Eight patients with normal gastric biopsy specimens had no PCR positive oral specimens or gastric aspirates. All, however, had PCR positive gastric biopsy specimens. In an attempt to determine the origin of these positive results in normal patients, it was shown that biopsy forceps could contaminate specimens with DNA from previous patients.

Conclusion—The demonstration of the organism in the mouths of a substantial proportion of dyspeptic patients has major implications for the spread of *H pylori* and identifies a potential source for reinfection following eradication of the organism from the stomach.

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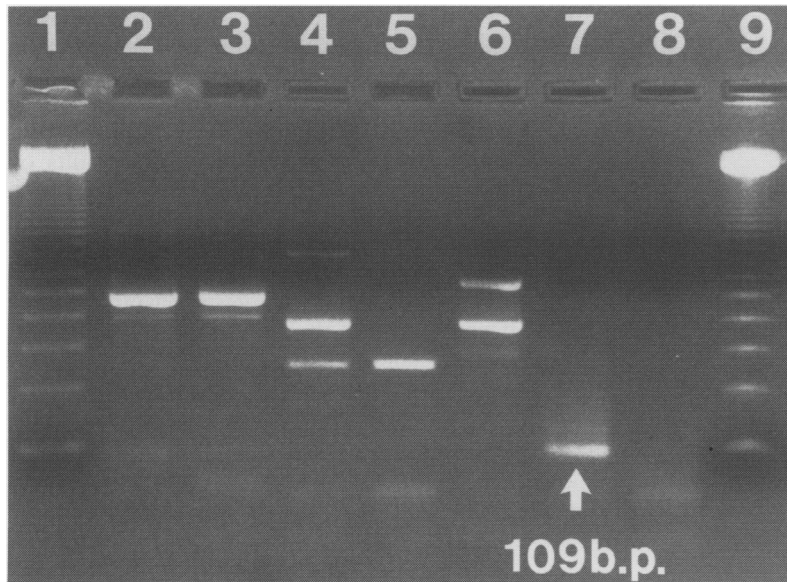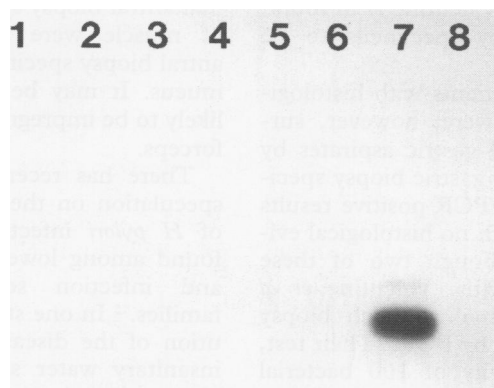Leeds, LS19 6BT

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Few data are available for the prevalence of *Helicobacter pylori* in the mouth. Information from conventional microbiological techniques varies. Majmudar *et al*, in a population of Indian patients, found a high prevalence with all 40 patients having positive oral specimens by culture and CLO test.[1] Another study, using the CLO test as the only indicator of oral infection, suggested an oral prevalence of 98% in dyspeptic patients. This included a significant number of patients with no evidence of gastric infection.[2] Krajden *et al* found a much lower prevalence, however, with only one of 71 patients having positive oral specimens by culture.[3] If the organisms are present in the mouths of patients with gastritis it could be a reservoir for spread of the disease. It would also be a likely source of reinfection, accounting for recurrence of Helicobacter gastritis and associated duodenal disease in 35·3% of patients with duodenal ulcer in the year following eradication of infection.[4]

Several reports have appeared in which molecular techniques, specifically the polymerase chain reaction (PCR), have been used for the identification of *H pylori*.[5–9] We recently described a PCR test for the organism[10] and sought to use it to clarify the prevalence of oral carriage of *H pylori*.

Methods

Twenty three dyspeptic patients had saliva taken before and after citric acid stimulation. Dental plaque was collected with a sterile toothpick. The patients then underwent upper gastrointestinal endoscopic examination. Gastric juice was aspirated through a sterile Wilson Cook catheter. Two gastric antral biopsy specimens were taken, one submitted for histological examination (including a modified Giemsa stain), and one for PCR. Endoscopes were fully disinfected using an Auto Disinfector II (Olympus Keymed) before and after each examination.

All PCR specimens were immediately frozen in individual containers and underwent DNA extraction using a standard proteinase K, hexadecyl-trimethyl-ammonium bromide (CTAB) and phenol chloroform method identical with that used in our initial work.[10]

Our PCR method differed from that used previously[10] in that a nested PCR was used to enhance sensitivity. Previous experiments had shown that a single step PCR detected *H pylori* in gastric specimens, but was not sensitive enough to show the presence of the organism in saliva.

Three primers were used; an initial 30 cycles with an annealing temperature of 55°C amplified a 446 base pair fragment of the gene encoding *H pylori* 16S rRNA. Two primers were used in this step—HP1 and HP3. A further 30 cycles with an annealing temperature of 62°C amplifying a 109 base pair fragment were then performed. This step used one primer from the initial reaction and another internal to the fragment amplified in that step (HP1 and HP2). The sequences of the primers (expressed 5' to 3') were as follows: *HP1* (CTG GAG AGA CTA AGC CCT CC, position 834–853); *HP2* (ATT ACT GAC GCT GAT TGT GC, position 744–763); and *HP3* (AGG ATG AAG GTT TAA GGA TT, position 407–426).

Using this method as little as 10 fg of bacterial DNA could be identified (corresponding to about 10 bacteria). The specificity was high, with no reaction to the most closely

Figure 1   PCR gel showing specificity of the test. Lanes 1 and 9 123 base pair ladder; lanes 2 and 3 0·1 µg H mustelae DNA; lane 4 0·1 µg H fennelliae DNA; lane 5 0·1 µg H cinaedi DNA; lane 6 0·1 µg Wolinella succinogenes DNA; lane 7 0·01 pg H pylori DNA; lane 8 negative control. 109 base pairs is the specific amplification product of H pylori DNA.

Histological and PCR results

Sample	Helicobacter positive patients (n = 13)	Histologically normal patients (n = 8)
Unstimulated saliva	1/13 positive	0/8 positive
Stimulated saliva	3/13 positive	0/8 positive
Tooth scrapings	2/13 positive	0/8 positive
Gastric aspirates	12/13 positive	0/8 positive
Gastric biopsies	12/13 positive	8/8 positive

related non-Helicobacter *Wolinella succinogenes*,[11] or the other Helicobacters known to be found in humans, *H fennelliae* or *H cinaedi*. There was a faint reaction when very large amounts of DNA from *H mustelae* were used (fig 1). This faint reaction was seen with 1×10^{-7} g of *H mustelae* DNA. As little as 1×10^{-14} g *H pylori* DNA was needed for a much stronger positive band. *H mustelae* has been cultured in ferrets but not in humans. Non-specific bands of amplified DNA of varying sizes were sometimes present in the nested PCR. These bands were seen only when there was no *H pylori* DNA in the specimen. They are probably due to non-specific amplified products from the increased number of cycles required to detect the very small numbers of bacteria.

Southern blots were performed on a selection of the PCR positive samples to confirm that the amplified product was from *H pylori*. It was also done on the specificity experiments to exclude cross-reactions. The PCR products were transferred to a nitrocellulose membrane. A P^{32} labelled internal probe was used (*pHP*, sequence 5' to 3' CAT CCA TCG TTT AGG GCG TG, position

806–825) This was hybridised with the PCR products on the membrane overnight at 45°C. Only the specific size band (109 base pairs) hybridised with the *H pylori* specific oligonucleotide probe (fig 2).

Each patient's group of five specimens underwent DNA extraction in a single batch accompanied by a negative control tube containing only water, but subjected to all the same steps as the other specimens. This controlled for contamination from the laboratory environment, or cross-contamination among an individual patient's specimens. Each PCR amplification was performed with a further negative control containing water instead of any specimen, and a positive control containing 100 fg of *H pylori* DNA. These controls excluded the possibility of positive reactions being due to contamination of PCR reagents, or negative reactions due to PCR inhibition. The PCR was performed in sterile conditions, with all DNA extraction, PCR setup, and post PCR analysis being done in three separate rooms.

Results

Fifteen of 23 patients had a Helicobacter associated gastritis on histological examination. Of these, two had contamination during extraction (shown by positive results in their extraction control specimens) and these patient's specimens were discarded (table). Of the remaining 13 patients, 12 had a PCR positive gastric aspirate specimen (fig 3) and 5 of them had at least one positive oral specimen.

The eight patients with histologically normal biopsy specimens had no positive oral or gastric aspirate specimens. All had faint PCR positive results for their antral biopsy specimens.

Figure 2   Southern Blot of specificity PCR shown in figure 1, showing hybridisation of probe with H pylori specimen alone.

Discussion

We used a nested PCR in this study rather than a one step PCR. We found that a one step PCR was not sensitive enough for our purposes. It had a good sensitivity for detecting *H pylori* in gastric biopsy specimens and aspirates, but no oral specimens were found to be positive. A nested PCR not only increases the sensitivity of a PCR reaction but also should increase the specificity of the reaction.

H pylori were shown in the mouths of five of 13 (38·5%) of patients with gastritis. Hammar *et al*, using a PCR test with a sensitivity of 20 copies of the target gene, detected
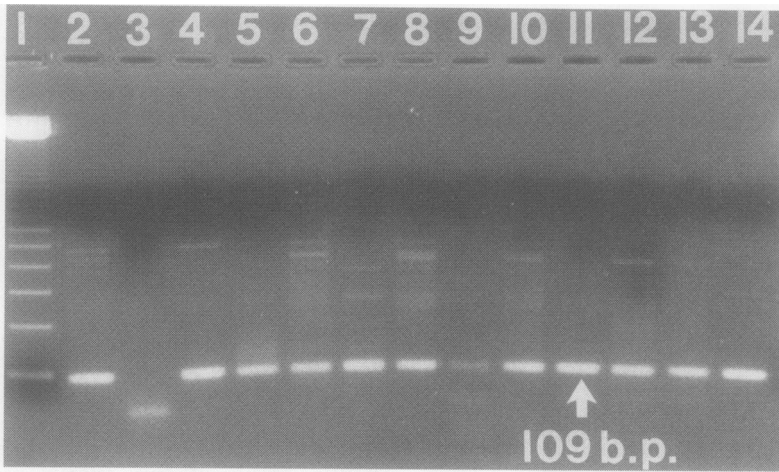
Figure 3   PCR gel of gastric aspirates from 13 patients all with Helicobacter gastritis. Lane 1 123 base pair ladder; lane 3 negative PCR result; lanes 2 and 4–14 positive PCR results.

the organism in nine of 19 (47·4%) patients with gastritis.[8] Both these results are at variance with European figures for oral carriage determined by culture, one of 71 (1·4%) positive,[3] and Indian figures of 40 of 40 (100%)[1] also determined by culture. The PCR results could represent the true oral prevalence of the organism in this country. If the mouth is a reservoir for reinfection, the low prevalence would explain why not all patients became reinfected after treatment. The patients in this study, however, were not ideal for the collection of oral specimens. All were due to undergo endoscopy, so many had cleaned their teeth immediately before arrival. This made collection of dental plaque particularly difficult. All had been fasting so very little saliva could be aspirated without citric acid stimulation. A similar study using PCR on patients not awaiting imminent endoscopy is planned.

The results for the gastric specimens in patients with gastritis were as expected, given the sensitivity of the test. All patients had at least one gastric specimen that was PCR positive. Twelve of 13 aspirates were PCR positive and 12 of 13 biopsy specimens were PCR positive. This compares with the figures from Clayton *et al*, using a PCR of similar sensitivity to the urease gene of *H pylori*.[7] They found that nine of 10 biopsy specimens from patients with histological evidence of *H pylori* were positive by PCR. Hammar *et al* found all of 15 patients positive for *H pylori* by culture were positive by PCR.[8] Valentine *et al* found 11 of 13 gastric biopsy specimens to be positive.[6]

The results for the patients with histologically normal stomachs were, however, surprising. All had negative gastric aspirates by PCR, yet *all* had positive gastric biopsy specimens. Clayton *et al* had PCR positive results in four of 11 patients with no histological evidence of *H pylori*,[7] although two of these patients did show gastritis. Valentine *et al* found none of 19 normal stomach biopsy specimens to be positive by PCR.[6] Their test, however, had a sensitivity of 100 bacterial cells, less than our test. They do not state

how they handled their specimens, whether they used all of a specimen or only a section. We digested and extracted the DNA from the whole biopsy specimen. They also do not state their criteria for initial diagnosis of specimens. Hammar *et al* only report results on patients with histologically confirmed gastritis.[8]

None of these eight patients had any other positive specimens. All of their oral specimens, and all of their gastric aspirates were negative. All of their controls were negative, making contamination of specimens during DNA extraction or PCR preparation very unlikely. One possible explanation for the PCR positive gastric biopsy specimens is that scanty *H pylori* (perhaps in its coccoid form) are present in normal stomachs. Loffeld *et al*, using immunohistochemical methods, reported *H pylori* in the gastric foveolae of 75% of patients with histologically normal gastric biopsy specimens.[12] This has not been confirmed by other workers. Alternatively, a non-pathogenic *Helicobacter* for which we have no specificity data could be present in normal stomachs. This explanation again seems flawed as none of these patients had positive gastric aspirates.

Alternatively, contamination could be occurring at the time of biopsy. The endoscopy equipment is mechanically cleaned and then disinfected using activated 2% glutaraldehyde. Although disinfection will kill the organism, it will not render the bacterial DNA refractory to amplification with PCR. Indeed, Karim *et al* reported culturable *Helicobacter* in one case following routine cleaning (though not disinfection) of biopsy forceps.[13] All our other specimens were collected with disposable equipment, except for the gastric aspirates, which were collected in sterilised equipment.

In an attempt to confirm this hypothesis, samples were taken from pieces of *H pylori* negative animal muscle tissue with the same biopsy forceps used in this study, after they had been cleaned and disinfected in the usual way. The samples were treated identically to the gastric biopsy specimens and nested PCRs performed. Of six samples taken in this way, one showed evidence of contamination by *H pylori* DNA. All controls were negative. This incidence of contamination from biopsy forceps is less than the incidence of PCR positive specimens seen in the histologically normal antral biopsy specimens, but the samples of muscle were obviously different from antral biopsy specimens, not being covered in mucus. It may be that they were thus less likely to be impregnated by DNA from biopsy forceps.

There has recently been a good deal of speculation on the possible routes of spread of *H pylori* infection. A high incidence is found among lower socioeconomic groups,[14] and infection seems to cluster within families.[15] In one study from Peru the distribution of the disease was associated with an insanitary water supply.[16] These and other observations have suggested a faecal-oral or

oral-oral route for the spread of this disease. Work on experimental animals has shown that transmission of Helicobacter related organisms occurs in animals with oral contact but not in animals with mainly faecal contamination, such as rats.[17] This has been taken as evidence for the oral-oral route for infection. The demonstration of the organism in the mouth is corroboration for this hypothesis.

In conclusion, we have shown that our PCR test is a sensitive and specific indicator of *H pylori* in gastric aspirates and have shown *H pylori* DNA in the mouths of five out of 13 patients with a Helicobacter associated gastritis. We have also shown that DNA contamination of specimens taken by reusable biopsy forceps can occur even if they are disinfected and washed. This has implications for all researchers using the PCR on specimens collected by this method, whether the site of biopsy be the gastrointestinal or other systems.

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