Evaluation of the polymerase chain reaction for detecting the urease C gene of *Helicobacter pylori* in gastric biopsy samples and dental plaque

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Summary. A polymerase chain reaction (PCR) assay with oligonucleotide primers homologous to a portion of the urease C gene of *Helicobacter pylori* was evaluated for specificity with pure DNA and biopsy material. The assay was used to test for the presence of the organism in dental plaque. The species specificity of detection was confirmed by ensuring that the primers did not amplify DNA extracts from *H. cinaedi*, *H. felis*, *H. fennelliae*, *H. mustelae* and *H. nemestrinae*. Sixty-two gastric biopsy samples collected from 14 patients (antrum, body and duodenal sites) were cultured and PCR was performed on the samples after culture. Primer sites were conserved in genomically diverse strains. Samples prepared by single-step heat lysis of bacterial cells and biopsy material did not inhibit PCR. The overall specificity was 96% irrespective of genotype. *H. pylori* was not cultured from dental plaque (15 patients), neither was *H. pylori* DNA detected by PCR in either urea breath test-positive or -negative individuals. The results showed that primer pair sequences within the urease C gene are conserved in most strains and provide an accurate basis for detecting *H. pylori*. As the PCR assay was not inhibited and did not yield false positive results with crude extracts from organisms or in the presence of biopsy material, its value as a diagnostic test was confirmed.

Introduction

Helicobacter pylori colonises the human gastro-duodenal mucosa, causes histological chronic active gastritis, is a co-factor in the development of duodenal ulceration and is associated with gastric ulceration and gastric cancer. Sero-epidemiological studies have indicated that *H. pylori* infection is common in both developed and developing countries.^{1,2} At present, man appears to be the principal host reservoir of *H. pylori* and its natural niche is the human stomach, although it is able to colonise gastric epithelial cells in the duodenum and oesophagus. *H. pylori* has not been cultured from other sites in the upper and lower gastro-duodenal tract, nor from faeces or saliva, but some isolations from the dental plaque of symptomatic and asymptomatic individuals have been described.^{3–5} Occasionally, gastric strains of *H. pylori* have been isolated from pig, baboon and rhesus monkeys; domestic and captive animals as well as birds appear to provide a wide host range for a diversity of other *Helicobacter* spp.

Because of the difficulty of culturing *H. pylori* from sites other than gastric mucosa, and the need for non-invasive diagnostic methods, particularly in the

investigation of acquisition of infections by young children, interest has grown in the use of molecular techniques for detection of this species. DNA probes for the detection of *H. pylori* in biopsy specimens have been described,^{6–8} and progress has been made by use of the polymerase chain reaction (PCR), which provides a specific and highly sensitive means of detecting microbial pathogens in clinical material. PCR assays have detected *H. pylori* DNA in clinical wax-paraffin-embedded^{9,10} and fresh gastric biopsy specimens,^{9–13} in faecal suspensions seeded with the organism,¹⁰ in faeces,¹⁴ in saliva,^{11,12,15,16} in gastric aspirates^{11,13,15} and in dental plaque.^{15,16}

In this study, a PCR assay based on amplification of a specific internal region of the urease C gene of *H. pylori* was used. Its specificity and reliability were evaluated for direct detection of the micro-organism in gastric biopsy samples, and the assay was applied to dental plaque to determine whether this site is a possible reservoir of *H. pylori*.

Materials and methods

Bacterial strains and culture media

The *H. pylori* strains used were NCTC 11637 (the type strain originating from Perth, Australia) and 14

Table I. Helicobacters used for testing specificity of the PCR assay with the urease C primer pair

Specific PCR product	No PCR product
<i>H. pylori</i> NCTC 11637 ^T	
<i>H. pylori</i> isolates (series 1):	<i>H. pylori</i> isolates (series 1):
1. (2a; Royal Free Hospital, London)	14. (10a; Royal Free Hospital, London)
2. (7a; Royal Free Hospital, London)	15. (10b; Royal Free Hospital, London)
3. (8a; Royal Free Hospital, London)	
4. (8b; Royal Free Hospital, London)	Other helicobacters:
5. (11a; Royal Free Hospital, London)	<i>H. mustelae</i> NCTC 12198 ^T
6. (12a; Royal Free Hospital, London)	<i>H. cinaedi</i> NCTC 12423 ^T
7. (13a; Royal Free Hospital, London)	<i>H. nemestrinae</i> NCTC 12491 ^T
8. (14a; Royal Free Hospital, London)	<i>H. fennelliae</i> NCTC 11612 ^T
9. (15a; Royal Free Hospital, London)	<i>H. felis</i> NCTC 12436 ^T
10. (16a; Royal Free Hospital, London)	
11. (87-141; USA)*	
12. (A601/90; Ipswich Hospital)*	
13. (A824/90; South Africa)	

^T Type strain.

* Heated cell lysates not tested.

clinical strains isolated from human gastric biopsy samples collected from patients (series 1) in the UK, USA and South Africa (table I). Clinical isolates (from the University Department of Medicine, Royal Free Hospital, London) were isolated from biopsy samples by inoculating specimens into selective enrichment broth (Difco Brucella Broth supplemented with fetal calf serum 10%; Isovitalex 1%; polymyxin B 10⁶ units/L; vancomycin 10 mg/L; amphotericin B 2 mg/L). Specimens were transported to the microbiology laboratory within 3 h. Each biopsy sample was then placed in 5 ml of selective enrichment medium in a 100 ml conical flask. The flasks were incubated at 37°C on a gyratory platform in a variable atmosphere incubator (Don Whitley Scientific Ltd, Shipley, Yorks) under micro-aerobic gaseous conditions (O₂ 5%, CO₂ 5%, H₂ 2%, N₂ 88%). After 48 h, a sample from each flask was subcultured on to Brain Heart Infusion Agar (Oxoid), supplemented with horse blood 5% and Isovitalex 1%. The isolates from the USA and South Africa were received as pure cultures.

Oral specimens, consisting of dental plaque and toothbrushings (plaque and saliva) were collected with dental toothpicks and toothbrushes, respectively, from another 15 patients (series 2) before treatment with a triple therapy regimen. The samples were collected by scraping interdental regions and subgingival crevices. All oral specimens were inoculated directly on to Skirrow's selective medium (blood agar base with vancomycin 10 mg/L; polymyxin B 2500 units/L, trimethoprim 5 mg/L) and nine were also incubated in selective enrichment broth as described above. Samples from the broth cultures were subcultured on to Skirrow's medium for examination of colony morphology. For this series of patients, the ¹³C urea breath test was performed by standard procedures.¹⁷

Isolates of *H. pylori* were identified by Gram's stain and colonial morphology, production of catalase and oxidase,¹⁸ and by production of urease.¹⁹ All isolates

were preserved at -196°C on glass beads in Nutrient Broth No. 2 (Oxoid) containing glycerol 10% v/v. Reference (taxonomic type) strains of the following five bacterial species closely related to *H. pylori* were used in the specificity studies: *H. mustelae* NCTC 12198 (strain R85-136P; ATCC 43772), *H. cinaedi* NCTC 12423 (strain CLO-165; ATCC 35683), *H. fennelliae* NCTC 11612 (strain CLO-231; ATCC 35684), *H. nemestrinae* NCTC 12491 (strain 15422) and *H. felis* NCTC 12436 (strain CS1).

Extraction of target DNA from cultures

Chromosomal DNA samples from *H. pylori* NCTC 11637 and clinical isolates of *H. pylori* (patient series 1), and the five other reference helicobacters were prepared with cetyltrimethylammonium bromide (CTAB) according to the DNA miniprep protocol of Wilson.²⁰

Preparation of biopsy samples for PCR

Antral, body and duodenal biopsy samples collected during the investigation of 14 patients with dyspepsia²¹ (series 3) were stored frozen at -70°C after incubation for 3 days. The sample set comprised 42 biopsy samples that gave positive results for *H. pylori* by conventional diagnostic tests (culture, histology and urease production) and 20 samples that gave negative results in those tests. To prepare each biopsy sample for PCR, 50 µl of sterile distilled water was added to the Eppendorf tube containing the biopsy sample and vortex mixed for c. 1 min. The samples were then boiled for 10 min, followed by centrifugation for 5 min. Ten µl of the resulting supernate was used in the PCR reaction as described below.

Preparation of dental plaque for PCR

Samples of dental plaque from five patients (series 4) attending clinics at the Royal Free Hospital and from

Fig. 1. PCR amplification of eight different strains of the genus *Helicobacter*. Lanes: 2–5, *H. pylori* NCTC 11637, 8b, 87-141 and A824/90 respectively; 6, *H. mustelae* NCTC 12198; 7, *H. cinaedi* NCTC 12423; 8, *H. nemestrinae* NCTC 12491; 9, *H. felis* NCTC 12436; 10, negative control; 1 and 11, 123-bp DNA ladder.

five asymptomatic subjects (all 10 patients and subjects in this series had undergone the urea breath test) were suspended in distilled water, and DNA was extracted by the CTAB method. Ten μ l of the resulting DNA solution was used in the PCR reaction as described below.

PCR primers

The oligonucleotide primer pair described by Brisou *et al.*²² as specific for two sequences flanking an internal 294 bp fragment of the urease C gene of *H. pylori*, was synthesised by standard procedures on an automated DNA synthesiser (Applied Biosystems Model 318A). These oligonucleotides were the forward primer (5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3') with 25 bases and a G+C content of 44 mol %, and the reverse primer (5'-AAGCTTACTTTCTAACACTA-ACGC-3') with 24 bases and a G+C content of 38 mol %.

PCR amplification

The template DNA was included in 100 μ l of a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, gelatin 0.01 % w/v, 0.2 mM of each deoxynucleotide (Pharmacia Ltd, Milton Keynes) and 0.5 μ M of each oligonucleotide primer. *Taq* DNA polymerase (2.5 units; Boehringer Mannheim, Poole) was added and the reaction mixture was overlaid with 100 μ l of mineral oil to prevent evaporation. PCR was performed on an Intelligent Heating Block (Hybaid Ltd, Twickenham, Middlesex). The amplification cycle consisted of an initial denaturation of target DNA at 99°C for 5 min followed by denaturation at 93°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. The final cycle included extension for 8 min at 72°C to ensure full extension of the product. Samples were amplified through 35 consecutive cycles. The completed reactions were analysed by submarine gel electrophoresis of a 15- μ l sample through agarose 1.0 % w/v and the bands were visualised after staining with ethidium bromide by excitation under UV light on a transilluminator. Negative controls consisted of a

“no DNA” reagent control and a “negative sample control”. The former contained all the necessary components except template DNA and the latter contained no target DNA sequence but had been subjected to all the sample preparation steps. To reduce the occurrence of false positive results, separate designated micropipettes were used for setting up reactions and for post-PCR work, reagents were divided into small single-use volumes, disposable gloves were worn, pre-mixtures were prepared, and the DNA was added last, as outlined by Kwok and Higuchi.²³ With each set of clinical PCR amplifications, a corresponding amplification was performed with *H. pylori* NCTC 11637 template DNA to provide a positive control.

Dot-blot hybridisation

Samples of amplification products obtained by PCR were confirmed by dot-blot hybridisation with the synthetic 44-base oligoprobe described by Brisou *et al.*²² which was complementary to a sequence internal to the region defined by the two PCR primers. The probe was labelled with biotin-dUTP by Klenow polymerase according to standard protocols²⁴ and was tested against a set of positive and negative PCR products as well as against positive (NCTC 11637 DNA) and negative (water) controls. The methods used for DNA dot hybridisation were described previously.²⁵

Results

Sensitivity of the PCR assay

Genomic DNA was extracted from *H. pylori* NCTC 11637 by the CTAB method, and the concentration was determined by measuring the optical density at 260 nm. Ten-fold serial dilutions were prepared from a starting concentration of 10 μ g/ml and were then subjected to PCR assay with urease C gene primers. The expected 294-bp PCR product was detected at a DNA template concentration of ≥ 100 pg for a reaction volume of 100 μ l. No *H. pylori*-specific product

Table II. Comparison of conventional diagnostic tests and PCR for detection of *H. pylori* in biopsy specimens

Patient (series 3)	Site of biopsy*	Culture and other tests†	R-type‡	PCR
A	b	+	1	+
A	a	+	1	+
A	d	+	1	+
A	b	+	2	+
A	a	+	1	+
A	d	+	1	+
B	a	+	3a	+
B	d	+	3b	+
B	b	+	3a	+
B	a	+	3c	+
B	b	—		—
C	d	—		—
C	a	—		—
C	b	—		—
C	a	—		—
C	d	—		—
D	d	—		—
D	a	—		—
D	b	—		—
D	b	—		—
D	d	—		—
D	a	—		—
E	a	+	4	+
E	d	+	4	+
E	b	+	4	+
E	a	+	4	+
E	d	+	4	+
E	b	+	4	+
F	a	+	5a	+
F	b	+	5a	+
F	d	—		—
F	a	+	5b	+
F	b	+	5a	+
F	d	—		—
G	d	—		—
G	b	+	6a	+
G	a	+	6b	+
G	b	—		—
G	a	+	6b	+
H	b	+	NT	+
H	d	+	NT	+
H	b	+	NT	+
H	a	+	NT	+
H	d	+	NT	+
I	a	+	7	+
I	a	+	7	+
J	d	+	NT	+
J	a	+	8	+
K	a	+	NT	+
K	a	+	NT	+
K	d	+	NT	+
K	b	+	NT	+
K	b	+	NT	+
L	b	+	9	+
L	b	+	9	+
L	a	+	9	+
L	d	+	9	+
L	a	+	9	+
M	a	—		—
M	a	—		—
N	a	—		—
N	a	—		—

* a, antrum; b, body; d, duodenum.

† Histology and urease test.

‡ *Hae*III ribopatterns; a, b, c indicate subtypes and NT, not typable with *Hae*III.²¹

was obtained with lower concentrations of template DNA.

Species specificity of the PCR assay

Genomic DNA was isolated by the CTAB method from a set of 15 assorted *H. pylori* isolates from patients in the UK (London and Ipswich), Australia, USA and South Africa, and five reference strains of other species of *Helicobacter*, and was tested in the PCR assay with the urease C gene primers. Results are listed in table I. The *H. pylori*-specific 294-bp PCR product was obtained with 13 of the 15 isolates of *H. pylori* and examples of electrophoresis of the product are shown in fig. 1. Two isolates (numbers 14 and 15), from gastric mucosa of the same patient (Royal Free Hospital, London), yielded no PCR product. A specific PCR product was not obtained with DNA of reference strains of *H. mustelae*, *H. cinaedi*, *H. nemestrinae*, *H. fennelliae* and *H. felis* (fig. 1). Identical positive and negative results were obtained when heated cell lysates were used for the PCR assay. The specificity of the PCR product was confirmed by DNA dot-blot hybridisation with the 45-base oligoprobe (results not shown).

Investigation of gastric biopsy samples

A total of 62 biopsy samples (antral and duodenal) from 14 patients (series 3) was tested in the PCR assay, and the results are listed in table II with previous data on conventional diagnostic tests and ribotyping. Some examples of the electrophoretic analysis of the PCR products obtained are shown in fig. 2. A summary of the PCR assay results is presented in table III with corresponding results for detection rates by direct culture. A total of 42 samples gave positive results by both methods (no false positive results), indicating 100% specificity for the PCR assay.

Investigation of dental plaque

All the oral specimens (24 toothpick samples and 24 toothbrush samples) from 15 patients (series 2) were inoculated on to Skirrow's selective medium. Toothpick and toothbrush specimens from nine of these patients were also incubated in selective enrichment broth. *H. pylori* was not isolated from any of the 48 oral specimens. Only the toothpick samples from six patient samples that were inoculated directly on to Skirrow's medium yielded colonies with a morphology resembling *H. pylori*. None of these cultures was urease positive or resembled *H. pylori* with Gram's stain. Nine oral cultures were purified to identify the micro-organisms by Gram's stain and a limited number of basic biochemical tests (growth at 37°C, production of oxidase and catalase). Examination of Gram's stains indicated the presence of small, as well as longer and thinner, gram-negative rods, and gram-positive rods with some cells in chains. The latter were

Fig. 2. PCR amplification of *H. pylori* from gastric biopsies. Lanes: 1, 123-bp DNA ladder; 2, positive control (NCTC 11637 DNA); 3–7, extracts from culture-positive biopsy samples; 8–12, extracts from culture-negative biopsy samples; 13, negative control.

Table III. Summary of PCR results for *H. pylori* in gastric biopsy samples compared with detection by direct culture (patient series 3)

PCR result	Culture result		Total
	+	–	
+	42	0	42
–	0	20	20
Total	42	20	62

Table IV. Summary of PCR results for *H. pylori* in dental plaque compared with urea breath test results (series 4)

PCR result	Breath test		Total
	+	–	
+	0	0	0
–	5	5	10
Total	5	5	10

Fig. 3. PCR amplification of dental plaque for detection of *H. pylori*. Lanes: 1, 123-bp DNA ladder; 2, positive control (NCTC 11637 DNA); 3–7, dental plaque from five individuals who were urea breath test-negative; 8, negative control.

Fig. 4. PCR amplification of dental plaque for detection of *H. pylori*. Lanes: 1, 123-bp DNA ladder; 2, positive control (NCTC 11637 DNA); 3–7, dental plaque from five individuals who were urea breath test-positive; 8, negative control.

thought to be lactobacilli, which are known to be resistant to vancomycin. They were the predominant organisms in the broth cultures and may have inhibited growth of other oral organisms. No further bacteriological identification of oral cultures was performed.

Ten dental plaque samples, comprising five from subjects with positive urea breath tests and five from subjects with negative urea breath tests were examined by PCR (figs. 3 and 4). The *H. pylori*-specific PCR product was not detected in any of these samples. However, most samples (9 of 10) yielded two common non-specific PCR product bands in the 861–984-bp region (e.g., lane 3, fig. 3). A summary of the PCR and breath test results is presented in table IV.

Discussion

In 1990, Gobert *et al.*¹¹ were the first to report that PCR of the urease gene region provided a sensitive and specific assay for detecting *H. pylori* in various types of clinical material—saliva, gastric aspirates and gastric biopsy samples. Our investigations were initiated with the *ureC* gene primers and internal oligoprobe defined by Gobert's group, although during the course of the work several other PCR assays based on different genomic regions were described for the detection of *H. pylori*.^{9,10,12–16} The results presented above confirm that these *ureC* gene primers are specific for *H. pylori*, and that at 55°C they did not amplify DNA from either *H. mustelae*, in accordance with Brisou *et al.*,²² or from four new species recently classified in the genus *Helicobacter*—*H. cinaedi*, *H. felis*, *H. fennelliae* and *H. nemestrinae*. An additional species, *H. muridarum*, was not available for comparison.

Evidence from DNA restriction digest analysis and rRNA gene patterns showed enormous genomic diversity in strains of *H. pylori* from different individuals.^{26–29} As there is evidence that polymorphisms also exist within the *ureC* gene,³⁰ and *ureA* and *B* genes³¹ of the urease region, one of the objectives of the present study was to test for conservation of the primer sites within a set of *H. pylori* isolates of defined genotypes from different patients and diverse locations. The sensitivity was 88% (14 of 16) when pure DNA or crude extracts were used for the PCR assay and 100% when 42 biopsy samples were used. The overall sensitivity was 96% (56 of 58) despite the fact that the *H. pylori* strains were genomically diverse, with nine different *Hae*III ribopattern types, seven subtypes and 11 untypable strains. The results showed that the *ureC* primer sites are usually conserved, but not in every strain. Pure DNA and crude lysates from isolates 14 and 15 gave consistently negative results in the PCR assay. Both isolates were from the same patient (antrum and body) and were identical in their ribopatterns and, as the PCR data suggest, were probably isogenic. The results indicate, therefore, that false negative results due to sequence divergence may occur occasionally when PCR based on the *ureC* gene

is used as a diagnostic tool for *H. pylori*. Furthermore, the results obtained with the crude lysates and biopsy samples showed that direct PCR of such material did not inhibit the amplification reaction.

Access to *H. pylori* at more accessible sites than the gastric mucosa would greatly facilitate investigation of infections, particularly in children. The possibility that *H. pylori* may colonise the oral cavity has attracted considerable attention, but positive isolates have been reported only from the dental plaque of 40 healthy volunteers in India,⁴ and from seven of 15 duodenal ulcer patients with *H. pylori*-associated gastritis and two of four patients with gastritis only in Scotland.⁵ Investigators in Canada cultured samples of dental plaque and saliva from 71 patients: only one dental plaque sample gave a positive culture for *H. pylori*.³ It is difficult to determine from the conflicting culture data whether saliva and dental plaque represent a significant reservoir of *H. pylori*, but our negative culture results are consistent with the Canadian study³ and those of other UK investigators (N. Batnavala, personal communication).

PCR provides an alternate approach to determine if *H. pylori* is present at low levels in the human oral cavity and studies in Sweden¹² and France¹¹ have reported detection in 48% and 70%, respectively, in saliva of patients. Detection rates in dental plaque were significantly lower (< 10%) and depended on the DNA regions targeted by the primers—for the *ureC* assay, only two of 124 samples gave positive results.¹⁶ Our negative results in detecting *H. pylori* DNA in dental plaque of a smaller series of 10 subjects—five of whom were infected in the gastroduodenum as indicated by the urea breath test—are consistent with the data of Birac *et al.*¹⁶ The negative isolation and PCR detection rates we have observed from oral samples suggest insignificant colonisation of dental plaque and add further evidence to the view that the presence of *H. pylori* in the mouth is transient. However, this hypothesis is contradicted by previously published results of some culture studies^{4,5} and of the PCR assays of saliva, which indicated a significantly higher level of positivity for *H. pylori*^{11,12,14–16} in the mouth. It is concluded that dental plaque may contain a factor that inhibits PCR or may affect its specificity, particularly with the *ureC* primers. For instance, PCR assays with primers based on the sequence of a species-specific antigen of *H. pylori* gave higher detection rates than *ureC* primers with dental plaque samples.¹⁶ Further studies are needed to clarify the apparent discrepancies between culture and PCR detection rates for *H. pylori* in the mouth of asymptomatic and infected individuals.

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