JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1991, p. 2543–2549
0095-1137/91/112543-07$02.00/0

Vol. 29, No. 11

Direct Polymerase Chain Reaction Test for Detection of *Helicobacter pylori* in Humans and Animals

SHENG-ANG HO,[1] JANE A. HOYLE,[1] FRASER A. LEWIS,[1] ALISON D. SECKER,[2] DEBRA CROSS,[1] NICHOLAS P. MAPSTONE,[1] MICHAEL F. DIXON,[1] JUDY I. WYATT,[3] DAVID S. TOMPKINS,[2] GRAHAM R. TAYLOR,[4] AND PHILIP QUIRKE[1]*

Department of Pathology, University of Leeds, Leeds LS2 9JT,[1] Department of Pathology, St. James's University Hospital, Leeds LS9 7TF,[3] Department of Microbiology, Bradford Royal Infirmary, Bradford BD9 6RJ,[2] and Yorkshire Regional DNA Laboratory, Clarendon Wing, Leeds General Infirmary, Leeds LS1 3EX,[4] United Kingdom

Received 7 January 1991/Accepted 21 August 1991

We designed a polymerase chain reaction (PCR) for amplifying the *Helicobacter pylori* gene encoding 16S rRNA. Primers for the specific detection of *H. pylori* were designed for areas of the 16S rRNA gene in which there is the least sequence homology between *H. pylori* and its closest relatives. The specificity of detection was confirmed by ensuring that the primers did not amplify DNA extracts from the campylobacters *H. cinaedi*, *H. mustelae*, and *Wolinella succinogenes*, which are the closest relatives of *H. pylori*, as determined by 16S rRNA sequencing. Serial dilution experiments revealed the detection of as little as 0.1 pg of DNA by PCR and 0.01 pg by nested PCR. *H. pylori* DNA was detected successfully in clinical paraffin-embedded and fresh gastric biopsy specimens from patients positive for the bacterium and also in fecal suspensions seeded with the organism. The DNA from the nonculturable coccoid form of *H. pylori* was also identified by the primers. Universal primers designed for highly conserved areas on the 16S rRNA gene enabled large amplification products to be produced for direct sequencing analysis. Gastric bacteria resembling *H. pylori* have been isolated from animals. DNA of these animal gastric bacteria amplified with *H. pylori*-specific primers yielded PCR products identical to those from human isolates of *H. pylori*, as confirmed by the use of a 20-base radiolabelled probe complementary to an internal sequence flanked by the *H. pylori*-specific primers. The results of PCR amplification and partial 16S rRNA gene sequence analysis strongly support the contention that the gastric organisms previously recovered from a pig, a baboon, and rhesus monkeys are *H. pylori*.

Helicobacter pylori is now established as the most common cause of gastritis (4), and recent evidence shows that successful eradication of the organism prevents the relapse of duodenal ulcers (29). Thus, diagnosis and treatment of *H. pylori* infection are now of growing importance in ulcer management.

A variety of noninvasive and invasive methods have been described for the detection of *H. pylori*. The former consist of serological tests (23) and ^{13}C or ^{14}C breath tests (9, 38). Invasive methods rely on endoscopy and biopsy to provide material for culturing (8), histological examination (10), or rapid urease testing (19). All of these techniques have disadvantages; namely, (i) they are too insensitive to confirm complete eradication of the organism after treatment, (ii) they all fail to detect the coccoid form of the organism (32), and (iii) they cannot definitively identify *H. pylori* from animal sources, in which closely related organisms may be found (16, 34). The development of a highly sensitive technique would overcome these problems and might permit the identification of the sources and routes of transmission of *H. pylori*.

The polymerase chain reaction (PCR) can amplify minute quantities of nucleic acid from a variety of sources, and primers which provide absolute specificity for a defined nucleic acid target can be designed. PCR has already been used to identify several bacterial species (18, 24, 36). In designing a method for *H. pylori* detection, we chose to amplify the gene encoding 16S rRNA because the organism was definitively distinguished from campylobacters and other bacterial genera and placed in a new genus on the basis of 16S rRNA studies (7, 15, 26, 30, 33). Also, sequences from several closely related bacteria are available and, if required, it should be possible to use the many thousands of copies of 16S rRNA per bacterium by including a preliminary reverse transcription procedure to enhance sensitivity. In addition, "universal" sequences for which primers could be designed to act as a positive control are present. This is important because false-negative results have been known to occur from the inhibition of amplification, especially in paraffin-embedded material (17). These primers could be used to ensure that any DNA extracted from negative control material is of suitable quality for use in PCR and that negative PCR results are not due to the influences of PCR inhibitors. Furthermore, a synthetic oligonucleotide probe complementary to a nucleotide sequence present in the 16S rRNA gene has recently been described for the specific detection of *H. pylori* (21).

A variety of gastric bacteria, most of which are morphologically and biochemically distinct from *H. pylori*, have been observed in animals. However, gastric organisms isolated from a pig and a baboon (13) and four rhesus monkeys (22) have proven to be remarkably similar to *H. pylori*. Using chain-terminating inhibitors, as described by Sanger and colleagues for direct sequencing of PCR products (31), we obtained partial 16S rRNA gene sequences for each gastric spiral organism recovered from a pig, a baboon, and two of the four rhesus monkeys to determine whether these animals were indeed infected by "human" *H. pylori*.

In this paper, we describe the development of a PCR assay for the sensitive and specific detection of *H. pylori*, together

* Corresponding author.

TABLE 1. Primer sequences

Primer	Sequence (5′ to 3′)	Region of 16S rRNA in reference to *E. coli*
U1	CGG TTA CCT TGT TAC GAC TT	1491–1510
U2	CCT TGT ACA CAC CGC CCG TC	1386–1405
U3	CAG CAG CCG CGG TAA TAC	518–535
U4	CAG TGG GGA ATA TTG CAC AA	354–373
Hp1	CTG GAG AGA CTA AGC CCT CC	834–853
Hp2	ATT ACT GAC GCT GAT TGT GC	744–763
Hp3	AGG ATG AAG GTT TAA GGA TT	407–426
pHp	CAT CCA TCG TTT AGG GCG TG	806–825

with the use of an internal probe and the direct sequencing of PCR products for confirmation of the specificity of the assay.

MATERIALS AND METHODS

Universal primer design. Highly conserved areas of the 16S rRNA gene were identified (40), and three sets of universal primers were generated to amplify fragments of 124 bp (U1-U2), 992 bp (U1-U3), and 1,156 bp (U1-U4) (Table 1). The universal primers had broad specificity for a range of eubacteria. All primers were constructed with a model 391 DNA synthesizer (PCR-MATE; Applied Biosystems, Foster City, Calif.) and 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite chemicals (Cruachem Ltd., Glasgow, United Kingdom).

Design of Hp1. Published sequences for *H. cinaedi*, *H. pylori*, *Wolinella succinogenes*, and the closest *Campylobacter* relatives, *Campylobacter fetus* subsp. *fetus*, *C. lari*, *C. jejuni*, *C. coli*, *C. sputorum* subsp. *sputorum*, and *Escherichia coli* (15, 26, 30, 33), were compared, and areas on the 16S rRNA genes showing the least homology were identified. Particular attention was paid to *W. succinogenes*, since it was the closest relative of *H. pylori* on the basis of available 16S rRNA sequences (33) and sequence information concerning *H. felis* and *H. mustelae* was inaccessible at the time. A segment from bases 834 to 853 of the 16S rRNA gene demonstrating a low level of homology was identified, synthesized, and called Hp1. This primer contains 10 base mismatches and a single base insertion when compared with *W. succinogenes*. The 16S rRNA sequences of *H. felis* and *H. mustelae* have recently been determined (27), and the sequence of primer Hp1 differs by 5 bases when compared with the sequence of *H. felis* and by 3 bases plus an insertion when compared with the sequence of *H. mustelae*. When used with U3, Hp1 is predicted to produce a PCR product of 335 bp.

Design of Hp2 and Hp3. The second *H. pylori*-specific primer (Hp2) was designed to eradicate weak amplification products identified with Hp1-U3. Areas of greatest mismatch of *H. pylori* with *C. lari*, *C. jejuni*, and *C. coli* were selected. A region from bases 744 to 763 of the 16S rRNA sequence was identified as having a low level of homology between these organisms. Information on *H. mustelae* and *H. felis* was not available at the time, but it was later ascertained that the sequence flanked by Hp2 was not homologous to the sequences of these species (see Discussion). Hp1 and Hp2 together amplified a 109-bp PCR product. A third primer (Hp3) was designed to enable nested PCR (25, 28) to be performed to a further area of low homology (bases 407 to 426) on the 16S rRNA sequence.

Bacteria tested. DNA was extracted from *H. pylori* (human clinical isolates and NCTC 11637), *H. mustelae* (F1 and F8, isolated by D. S. Tompkins), *H. cinaedi* (Seattle 48), *C. fetus* subsp. *fetus* (NCTC 10348), *C. lari* (NCTC 11352), *C. jejuni* (NCTC 11168), *C. coli* (NCTC 11353), *C. sputorum* subsp. *sputorum* (NCTC 11528), *Escherichia coli* (JM101), and *W. succinogenes* (NCTC 11488). A 0.7% agarose electrophoresis gel stained with ethidium bromide was used for qualitative analysis, and the DNA was quantified by use of a TKO-100 dedicated minifluorometer (Hoefer Scientific Instruments, San Francisco, Calif.) and the fluorochrome Hoescht 33258 (Polysciences Inc., Warrington, Fla.).

Pig, baboon, and rhesus monkey isolates. Bacteria which were isolated from the stomachs of a laboratory pig and a laboratory baboon and which had been previously characterized (13) were generously donated by D. M. Jones (Public Health Laboratory, Manchester, United Kingdom). Two isolates from rhesus monkeys were kindly donated by D. Newell (Public Health Laboratory Service, Porton Down, Salisbury, United Kingdom) (22). DNA was extracted, and an aliquot of the extract was amplified with Hp1 and Hp2 at an annealing temperature of 60°C for 40 cycles.

DNA extraction. Bacterial DNA was extracted by suspending colonies from one plate in 567 μl of TE buffer (10 mM Tris-Cl [pH 7.5], 1 mM EDTA) in a 1.5-ml Eppendorf tube. To this was added 30 μl of 10% sodium dodecyl sulfate (SDS; Sigma Chemical Co. Ltd., Poole, Dorset, United Kingdom) and 3 μl (2 mg/100 μl) of proteinase K (BRL Life Technologies Inc., Gaithersburg, Md.). After the ingredients were mixed, the tube was incubated at 37°C for 1 h. Then, 100 μl of 5 M NaCl and 80 μl of hexadecyltrimethyl ammonium bromide (Sigma) were added, and the mixture was incubated at 65°C for 10 min (39). The contents were extracted with an equal volume of chloroform-isoamyl alcohol and centrifuged at 9,000 × *g* in a microcentrifuge for 3 min, and the aqueous layer was transferred to a fresh Eppendorf tube. Two further extractions were performed with equal volumes of phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol. The DNA was precipitated with 0.6 volume of isopropanol at −20°C. The DNA was pelleted by microcentrifugation at 16,000 × *g* for 5 min, washed with −20°C 70% (vol/vol) ethanol, desiccated for 30 min, and dissolved in 100 μl of molecular biology-grade water (BDH Ltd., Poole, Dorset, United Kingdom). The DNA was quantified as described above, and a 0.7% agarose electrophoresis gel was used for qualitative analysis.

Preparation of coccoid forms. A suspension of the nonculturable coccoid form of *H. pylori* was kindly supplied by H. Oosterhom (Gist-Brocades, Delft, Holland), and DNA was extracted by the above-described method.

DNA extraction from paraffin-embedded material. A standard proteinase K-SDS extraction protocol (6), modified by an increased proteinase K incubation time of 5 days (12), was used. A minimum of 10 4-μm sections were used, depending on the sizes of the gastric biopsy specimens. Care was taken during section preparation to prevent contamination from the microtome blade by cleaning the blade with xylene before and after cutting. DNA was assessed as described above and amplified by PCR.

PCR conditions. PCR was performed under the following conditions. One microliter of each oligonucleotide primer was placed (50 pmol/μl for each primer) in an Eppendorf tube and briefly vacuum desiccated, and 1 μl of extracted DNA, 5 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris-Cl, 15 mM $MgCl_2$, 0.1% [wt/vol] gelatin [pH 8.3]), 8 μl of deoxynucleoside triphosphate mixture (final concentration, 1.25 mM each dATP, dCTP, dGTP, and dTTP; BRL

Life Technologies or Pharmacia, LKB, Uppsala, Sweden), 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.), and molecular biology-grade distilled water (BDH) were added to make a final reaction volume of 50 μl. The tube was sealed with 40 μl of liquid paraffin, briefly spun in a microcentrifuge, and placed in a thermal cycler (Autogene; Grant Instruments, Barrington, Cambridge, United Kingdom). The temperature profile was as follows: 30 s at 95°C, 30 s at 55 or 60°C, and 30 s at 72°C. The last cycle was identical, except that the 72°C extension period was increased to 5 min and the mixture was subsequently refrigerated at 4°C before analysis. The number of cycles was 20, 30, or 40. For nested PCR, 25 cycles were used for each round of amplification. PCR products were analyzed on a 2% agarose electrophoresis gel stained with ethidium bromide.

Assessment of sensitivity. Tenfold serial dilutions of *H. pylori* DNA were amplified with Hp1 and Hp2, from a concentration of 1 ng/μl to 1 fg/μl. One Eppendorf tube containing distilled water (BDH) only was also included to exclude PCR reagent contamination. Annealing was done at 60°C for either 30 or 40 cycles. For nested PCR, Hp1-Hp3 was used for the first reaction and Hp1-Hp2 was used to amplify 1 μl of the reaction product from the first reaction. The sensitivity of nested PCR was also assessed.

Exclusion of cross-reactivity. To identify possible cross-reactions with other enterobacteria and to confirm the potential for fecal identification of *H. pylori*, we suspended human feces from a presumed *H. pylori*-negative child (4 years of age) in phosphate-buffered saline and seeded the suspension with known quantities of *H. pylori* (1:10 serial dilutions). The DNA was extracted and amplified with Hp1-Hp2. A negative control of feces only was also included.

Design of an oligonucleotide probe internal to the PCR product of Hp1-Hp2. A sequence from bases 806 to 825 of the 16S rRNA gene that exhibited several base mismatches between *H. pylori* and its closest relatives was identified. A probe was synthesized for this region with the aid of the model 391 DNA synthesizer and was called pHp. In this region, there is a single base difference between *H. pylori* and *H. cinaedi*, *H. fennelliae*, *H. mustelae*, and *W. succinogenes* (15, 27, 33). Up to 3 base mismatches can be identified between *H. pylori* and the campylobacters (15, 33).

Dot blot assay. The oligonucleotide probe pHp was labelled at the 3′ end with terminal deoxynucleotidyltransferase (Life Technologies Ltd., Paisley, United Kingdom) in the presence of [γ-^{32}P]dATP at 37°C for 2 h. Unincorporated dATP was removed by purification of the labelled probe on a Nensorb 20 cartridge (DuPont, Stevenage, Hertfordshire, United Kingdom). The gel used for analyzing the PCR products was blotted for 24 h onto nitrocellulose. The nitrocellulose blot was hybridized by use of a Hybrid 11 blot processing system (Hybrid Ltd., Teddington, United Kingdom) at 42°C overnight with 200 ng of radiolabelled probe pHp in a hybridization buffer containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, and 0.2% bovine serum albumin. The hybridization buffer was supplemented with 1.5 mg of salmon sperm single-stranded DNA (ssDNA) and 10 mg of yeast tRNA per 100 ml. After hybridization, the blot was washed sequentially with 500 ml of 2× SSC and 500 ml of 1× SSC at room temperature to remove excess and nonspecifically bound probe. The washed blot was dried, wrapped in cling film, and exposed to Rapid X X-ray film (GRI Ltd., Dunmow, Essex, United Kingdom) at −80°C for 6 h.

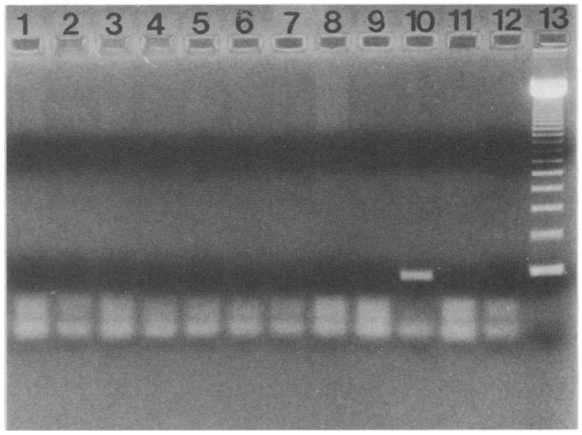

FIG. 1. PCR of *H. pylori* and its closest relatives with an annealing temperature of 60°C and 40 cycles of amplification. The PCR products were visualized on a 2% agarose electrophoresis gel stained with ethidium bromide to demonstrate the specificity of primers Hp1 and Hp2. Lanes: 1, *H. mustelae* F1; 2, *H. cinaedi*; 3, *C. fetus* subsp. *fetus*; 4, *C. lari*; 5, *C. jejuni*; 6, *H. mustelae* F8; 7, *C. coli*; 8, *C. sputorum* subsp. *sputorum*; 9, *E. coli*; 10, *H. pylori*; 11, *W. succinogenes*; 12, negative control; 13, 123-bp DNA ladder.

DNA sequencing. For the human isolates of *H. pylori* and the pig and baboon isolates, double-stranded DNA (dsDNA) was generated with primers U1 and U3 to produce a 992-bp fragment under the conditions described above but for 30 cycles with an annealing temperature of 55°C and extension for 1 min. Asymmetric PCR with 1 μl of Hp1 (1 pmol/μl) and 1 μl of U4 (50 pmol/μl) was also performed to generate ssDNA for sequencing. Asymmetric PCR was also performed under identical conditions with DNA extracted from both isolates from the two rhesus monkeys. The DNA generated was purified with a Centricon-30 column (Amicon Ltd., Gloucester, United Kingdom), and the DNA was sequenced by the dideoxy chain termination method of Sanger et al. (31) incorporating [^{35}S]dATP (1,300 Ci/mmol; NEN) and using modified T7 DNA polymerase (Sequenase; United States Biochemicals, Cleveland, Ohio). The sequencing primer was Hp1, and the molar primer/template ratio was 10:1. Sequencing reactions were assessed by 8% polyacrylamide gel electrophoresis.

RESULTS

Universal primers. The paired universal primers (U1-U2, U1-U3, and U1-U4) successfully amplified DNA to generate 124-, 992-, and 1,156-bp fragments from *E. coli*, *H. pylori*, and the pig, baboon, and rhesus monkey isolates. The larger fragments were used for DNA sequencing (see below). The universal 124-bp primer also successfully amplified DNA from all the bacteria included in the screening group. Thus, the 16S rRNA gene could be amplified by PCR in all of the samples under study, and we could exclude inhibition of PCR as a cause of negative results.

Hp1-U3. When all the bacterial DNA, except that from the pig, baboon, and rhesus monkey isolates, was amplified by the combination of 30 cycles and an annealing temperature of 55°C, only *H. pylori* DNA was amplified strongly. Weak amplification of *H. mustelae* F1 and F8, *C. lari*, *C. jejuni*, and *C. coli* DNA occurred. The weakly cross-reactive bands

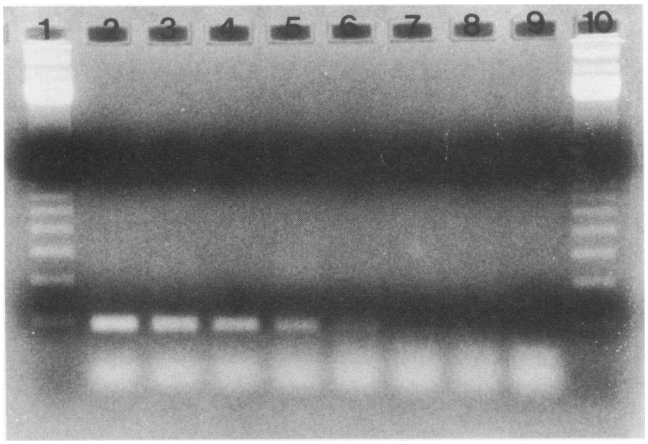

FIG. 2. Two percent agarose electrophoresis gel demonstrating the sensitivity of *H. pylori* detection with Hp1-Hp2 at an annealing temperature of 60°C and with 40 cycles of amplification. Lanes: 1 and 10, 123-bp DNA ladder; 2 to 8, *H. pylori* DNA at 1 ng (lane 2), 0.1 ng (lane 3), 0.01 ng (lane 4), 1 pg (lane 5), 0.1 pg (lane 6), 0.01 pg (lane 7), and 1 fg (lane 8); 9, negative control.

could be abolished by reducing the number of cycles to 20 or by increasing the annealing temperature to 60°C.

Hp1-Hp2. When Hp1-Hp2, 40 cycles, and an annealing temperature of 60°C were used, only *H. pylori* DNA yielded a 109-bp product (Fig. 1). The DNA from the closest relatives of *H. pylori* was not amplified. Thus, to ensure stringent specificity without compromising sensitivity, we used 40 cycles and an annealing temperature of 60°C in all PCR amplifications with Hp1-Hp2.

Nested PCR. When Hp1-Hp3 and 25 cycles followed by Hp1-Hp2 and 25 cycles were used, stronger amplification of the 109-bp final product was achieved.

Sensitivity. DNA amplification with Hp1-Hp2 for 30 cycles permitted the detection of 10 pg of starting bacterial DNA. Increasing the number of cycles to 40 improved the sensitivity of detection to 0.1 pg of DNA (Fig. 2, lane 6); with nested PCR, 0.01 pg of DNA was detectable.

Confirmation of the amplified product. Direct sequencing of an ssDNA PCR product, which was 499 bases long, from Hp1-U4 on *H. pylori* DNA provided 275 bases of readable sequence information. Sequencing of the 992-bp dsDNA PCR product from U1-U3 was also performed, and the 235 bases of readable sequence information obtained was identical to that from the ssDNA. However, we identified several sequence differences between the clinical isolate of *H. pylori* sequenced by us and that reported by Lau and colleagues (NCTC 11638) (15). By convention, positions of bases in the 16S rRNA sequence are numbered with reference to *E. coli*. We found one base mismatch (position 761), two deletions (between positions 701 and 702 and positions 703 and 704), and one insertion (between positions 670 and 671). On the basis of the partial sequence information that we have obtained, there is >97.3% homology between our *H. pylori* isolate and NCTC 11638.

Amplification of nucleic acid from coccoid forms of *H. pylori*. An extract of *H. pylori* DNA from nonculturable coccoid forms yielded a 109-bp product, confirming successful amplification. Annealing was done at 60°C for 40 cycles. The negative control from the DNA extraction protocol was not amplified, indicating that there was no extraction or PCR reagent contamination.

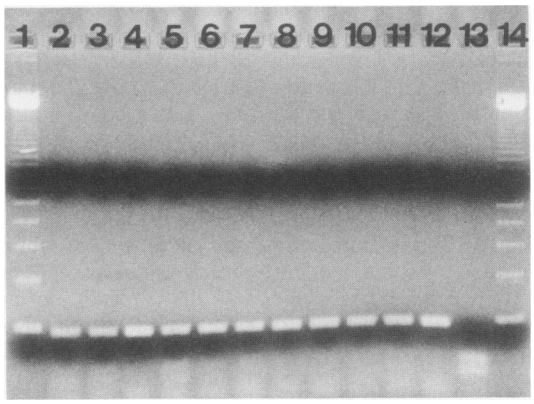

FIG. 3. Two percent agarose electrophoresis gel showing the detection of *H. pylori* in paraffin-embedded gastric biopsy specimens. Annealing was done at 60°C for 40 cycles. Lanes: 1 and 14, 123-bp DNA ladder; 2 to 11, PCR products from gastric biopsy specimens; 12, positive control; 13, negative control.

Paraffin-embedded and fresh materials. All 10 paraffin-embedded gastric biopsy specimens previously shown to have histologically verified *H. pylori* infection produced a 109-bp fragment (Fig. 3). Also, DNA amplified by Hp1-Hp2 from fresh gastric biopsy specimens obtained by endoscopy from patients with histologically verified *H. pylori*-associated gastritis produced a 109-bp fragment (Fig. 4, lane 4), whereas DNA from biopsy specimens from histologically normal gastric mucosa failed to be amplified (Fig. 4, lane 5). Tissues obtained postmortem from *H. pylori*-positive patients were also amenable to amplification (Fig. 4, lanes 2 and 3). Negative control tubes for the extractions were also included to rule out reagent contamination. Negative control samples from other human tissues (brain, kidneys, liver, spleen, lungs, and tonsils) did not produce any positive reaction, indicating that the *H. pylori*-specific primers did not amplify human DNA. Factor VIII primers (14) were used to confirm that the DNA from the negative control samples

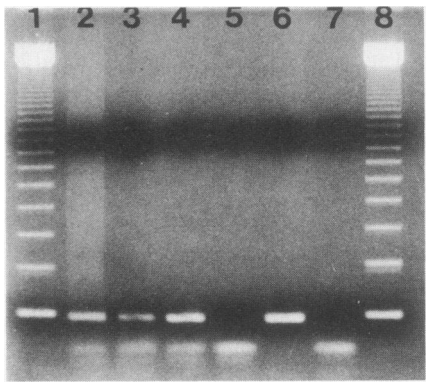

FIG. 4. Two percent agarose electrophoresis gel of the 109-bp PCR product from fresh tissue. Lanes: 1 and 8, 123-bp DNA ladder; 2, postmortem gastric biopsy specimen from an *H. pylori*-positive patient; 3, postmortem duodenal biopsy specimen from an *H. pylori*-positive patient; 4, gastric biopsy specimen obtained by endoscopy from an *H. pylori*-positive patient; 5, gastric biopsy specimen obtained by endoscopy from an *H. pylori*-negative patient; 6, positive control (*H. pylori* DNA); 7, negative control. Annealing was done for 40 cycles at 60°C.

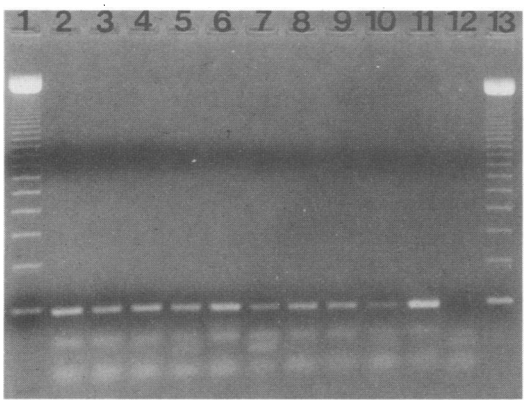

FIG. 5. Amplification of the 109-bp PCR product from feces seeded with twofold serial dilutions of *H. pylori* by 40 cycles of amplification at an annealing temperature of 60°C. Lanes: 1 and 13, 123-bp DNA ladder; 2 to 10, seeded fecal samples starting from 1.89×10^7 organisms per ml of fecal suspension from lane 2 on; 11, positive control (*H. pylori* DNA); 12, negative control (fecal suspension without any *H. pylori* added).

was amplifiable by PCR and to exclude false-negative results caused by *Taq* inhibition. An extraction reagent control and a distilled water control were also included.

Exclusion of cross-reactivity. DNA extracted from human feces alone failed to be amplified (Fig. 5, lane 12), while DNA from feces seeded with *H. pylori* at 1.89×10^7 to 7.40×10^4 organisms per ml yielded a 109-bp fragment (Fig. 5, lanes 2 to 11). A similar experiment was performed with the addition of serial dilutions of *H. pylori* DNA to 1 μg of human genomic DNA to exclude a nonspecific cross-reaction with human DNA. Amplification only occurred in samples containing added *H. pylori* DNA down to a concentration of 1 pg, at which the concentration ratio of *H. pylori* DNA to human genomic DNA was $1:10^6$. These experiments also showed that *H. pylori* DNA can be sensitively detected even when there is a large background of "foreign" DNA present.

Pig, baboon, and rhesus monkey isolates. When tested with Hp1-Hp2, the animal gastric isolates yielded a 109-bp fragment, indicating that extensive sequence homology in this area must exist. This supposition was confirmed by hybridization of the internal probe, pHp, to these PCR products. Probe pHp did not hybridize to PCR products from *H. cinaedi* or *H. mustelae* but did hybridize to amplified *H. pylori* DNA, indicating that positive blots were specific for *H. pylori* sequences only.

To provide more conclusive evidence that these animal isolates were indeed *H. pylori*, ssDNA sequencing and dsDNA sequencing were performed. A readable sequence of 275 bases was obtained from the organisms by ssDNA sequencing, and one of 235 bases was obtained by dsDNA sequencing. One base mismatch was identified for the pig isolate at position 761 on the 16S rRNA sequence, whereas the baboon and rhesus monkey isolates yielded sequences identical to *H. pylori* sequences over the whole length of the readable sequence. The level of homology between a human isolate of *H. pylori* and the pig, baboon, and rhesus monkey isolates over this 275-base region of 16S rRNA was >99.5%, providing strong evidence that these animal gastric isolates were human *H. pylori*.

DISCUSSION

For PCR amplification of *H. pylori*, two targets appear promising, the urease and 16S rRNA genes, because partial or whole sequence information is available for both (3, 15, 26, 30, 33). PCRs for the identification of a range of bacteria have recently been reported; some use the 16S rRNA gene as their target (2, 11). rRNA is a common but distinctive cellular component, and 16S rRNA-based sequencing methods have been shown to be highly suitable for demonstrating phylogenetic diversity in bacteria (5, 26, 30, 33, 37). Conversely, we did not target the urease gene, since only limited sequence information was available concerning other urease-producing bacteria when this project commenced. Additionally, specific primer design might have been difficult, because a recent DNA probe hybridization analysis has shown that urease genes exhibit conserved sequences among phylogenetically distant gram-negative bacteria (1). Particular difficulty might have occurred in the amplification of closely related urease-producing bacteria, such as *H. mustelae* and *H. felis*, or other gastric organisms, such as *Gastrospirillum hominis* (20).

We designed a PCR for *H. pylori* DNA amplification which is highly sensitive and specific by using the gene encoding 16S rRNA. We also showed that it is possible to obtain a high degree of sensitivity and specificity by using only one specific primer together with a universal primer if the annealing temperature is relatively high (60°C) or the number of cycles in the thermal profile is reduced. Thus, 16S rRNA should provide a versatile target for bacterial PCRs, since considerable sequence information is available, and specific amplification can be achieved even when there are relatively few sequence mismatches. We went on to design second and third primers, since high sensitivity and specificity were major requirements. This paper is the first to describe the amplification of *H. pylori* from paraffin-embedded material and also the firm identification of coccoid forms of this organism.

The use of factor VIII primers is a new approach to confirming the quality of the extracted material from negative control tissue blocks for DNA amplification, and universal 16S rRNA primers have been used for similar purposes by Malloy et al. (18). Such techniques serve to exclude the possibility of *Taq* DNA polymerase inhibition and the presence of very-low-quality unamplifiable DNA from invalidating any negative PCRs, especially with material such as paraffin-embedded tissue, which is less amenable to amplification (17, 41). It is essential to validate the quality of the material and its successful amplification to rule out false-negative results when using PCR tests in a clinical setting. These primers are also useful in indicating reagent contamination.

Our PCR with Hp1-Hp2 could detect as little as 0.1 pg of *H. pylori* DNA, representing the detection of about 100 bacteria, the same level of sensitivity reported by another group using PCR to investigate *H. pylori* (35). However, by increasing the number of cycles or by using nested PCR, we were able to enhance the sensitivity of our PCR to a detection limit of 0.01 pg of DNA. None of the existing *H. pylori* detection methods can approach this level of sensitivity. We did not obtain increased sensitivity by using reverse transcription (unpublished observation), as has been reported by other workers investigating *Mycobacterium tuberculosis* (2), but modification of our procedures is in progress.

We initially obtained weak cross-reactions with *H. mustelae*, *C. lari*, *C. jejuni*, and *C. coli* with Hp1-U3, but after

elevation of the annealing temperature from 55 to 60°C, only *H. mustelae* produced a band. In the area of the 16S rRNA gene to which primer Hp1 hybridizes, *H. mustelae* has three base mismatches and one base deletion, compared with *H. pylori*, whereas *H. felis* has five base mismatches (27). However, we were unable to investigate the latter organism, as bacterial DNA was not available for amplification. Cross-reactions with *H. mustelae* were later eliminated by use of Hp2. In the area of the 16S rRNA gene to which primer Hp2 hybridizes, *H. pylori* has one base mismatch and one base deletion compared with *H. mustelae* and two base mismatches compared with *H. felis*. The latter organism is unlikely to be recognized by this PCR if *H. mustelae* DNA fails to be amplified. No cross-reactions were experienced when human genomic or fecal DNA extracts were subjected to PCR. With the development of a third *H. pylori*-specific primer (Hp3), we were able to use nested PCR, further reducing the risk of cross-reaction with other bacterial DNA and removing the problem of concatemerization when studying archival material (25, 28).

Hoshina et al. also used 16S rRNA sequences for the specific detection of *H. pylori* (11). The primers that they described are not from the same region of the 16S rRNA gene as our primers, but it is not possible to determine the exact sites of their *H. pylori*-specific primers on the 16S rRNA gene sequence from their published data. This is because they did not state the regions of the 16S rRNA sequences to which their primers hybridized, in accordance with the convention of using *E. coli* for reference purposes. The specificity of *H. pylori* detection reported by Hoshina and colleagues is similar to that achieved with Hp1-Hp2. It is interesting that the annealing temperatures used by this group to achieve specific detection are also similar to ours, but Hoshina et al. only used a maximum of 30 cycles of amplification (11). We suspect that this fact may have compromised their sensitivity of detection, as our observations show that increasing the number of cycles from 30 to 40 greatly enhances the sensitivity of *H. pylori* detection without altering the specificity (as shown in Fig. 1). Unfortunately, Hoshina et al. did not express their sensitivity of detection in terms of the number of bacteria or amounts of DNA that could be amplified; thus, valid comparisons cannot be made with our assay or that of Valentine et al. (35).

Previous reports of animal infection indicated that *H. pylori*-like organisms were isolated from a pig, a baboon, and rhesus monkeys. Although these organisms were morphologically and biochemically similar to *H. pylori*, definitive proof of their identity was lacking. The sequence information reported here, the positive PCR amplification with Hp1-Hp2, and the hybridization with an internal probe are the best evidence to date that the organisms from the pig, the baboon, and the rhesus monkeys were indeed *H. pylori* and strongly suggest that these animals acquired their infections from human sources in the laboratory. Further investigation of such transmission is required to ascertain the frequency with which non-gnotobiotically reared animals can be infected with *H. pylori* and used as laboratory models of *H. pylori* infection.

The ability to detect *H. pylori* in feces offers the potential for a noninvasive test for infection and, if fecal detection is confirmed in *H. pylori*-positive subjects, would lead to considerable support for fecal-oral spread as the principal mode of transmission. The high sensitivity of such a test will be particularly valuable in searching oral bacterial niches and for detecting small numbers of bacteria which may persist in the stomach after therapy. While recrudescence of infection suggests the persistence of bacteria, such bacteria may remain undetected by available methods and give rise to false-negative results. Their detection is important, because the proliferation of residual organisms leads to reactivation of chronic gastritis and renders the patient liable to ulcer relapse. Thus, the confirmation of complete eradication of *H. pylori* by the PCR test will be of considerable value in patient management.

ACKNOWLEDGMENTS

We thank D. M. Jones (Public Health Laboratory, Manchester, United Kingdom) for providing bacterial specimens of pig and baboon gastric organisms, D. Newell (Public Health Laboratory Service, Porton Down, Salisbury, United Kingdom) for supplying two gastric isolates from rhesus monkeys, and H. Oosterhom for providing the coccoid forms of *H. pylori*. Thanks are also due to J. Fearnley for help in preparing the manuscript.

We also express our gratitude to Gist-Brocades for funding this project, which was also supported by an Intercalated B.Sc. grant from the Medical Research Council.

REFERENCES

1. **Blanchard, A., and M. F. Barile.** 1989. Cloning of *Ureaplasma urealyticum* DNA sequences showing genetic homology with urease genes from gram-negative bacteria. Res. Microbiol. **140**:281–290.
2. **Böddinghaus, B., T. Rogall, T. Flohr, H. Blöcker, and E. C. Böttger.** 1990. Detection and identification of mycobacteria by amplification of rRNA. J. Clin. Microbiol. **28**:1751–1759.
3. **Clayton, C. L., M. J. Pallen, H. Kleanthous, B. W. Wren, and S. Tabaqchali.** 1990. Nucleotide sequence of two genes from *Helicobacter pylori* encoding for urease subunits. Nucleic Acids Res. **18**:362.
4. **Dixon, M. F.** 1990. Progress in the pathology of gastritis and duodenitis. Curr. Top. Pathol. **81**:1–40.
5. **Estrada, I. C. E., F. I. Lamb, M. J. Colston, and R. A. Cox.** 1988. Partial nucleotide sequence of 16S ribosomal RNA isolated from armadillo-grown *Mycobacterium leprae*. J. Gen. Microbiol. **134**:1449–1453.
6. **Goelz, S. E., S. R. Hamilton, and B. Vogelstein.** 1985. Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. Biochem. Biophys. Res. Commun. **130**:118–126.
7. **Goodwin, C. S., J. A. Armstrong, T. Chilvers, M. Peters, M. D. Collins, L. Sly, W. McConnell, and W. E. S. Harper.** 1989. Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. nov. and *Helicobacter mustelae* comb. nov., respectively. Int. J. Syst. Bacteriol. **39**:397–405.
8. **Goodwin, C. S., E. D. Blincow, J. R. Warren, T. E. Waters, C. R. Sanderson, and L. Easton.** 1985. Evaluation of cultural techniques for isolating *Campylobacter pyloridis* from endoscopic biopsies of gastric mucosa. J. Clin. Pathol. **38**:1127–1131.
9. **Graham, D. Y., P. D. Klein, D. J. Evans, D. G. Evans, L. C. Alpert, A. R. Opekun, and T. W. Boutton.** 1987. *Campylobacter pylori* detected noninvasively by the ^{13}C-urea breath test. Lancet **i**:1174–1177.
10. **Gray, S. F., J. I. Wyatt, and B. J. Rathbone.** 1986. Simplified techniques for identifying *Campylobacter pyloridis*. J. Clin. Pathol. **39**:1279–1280.
11. **Hoshina, S., S. M. Kahn, W. Jiang, P. H. R. Green, H. C. Neu, N. Chin, M. Morotomi, P. LoGerfo, and I. B. Weinstein.** 1990. Direct detection and amplification of *Helicobacter pylori* ribosomal 16S gene segments from gastric endoscopic biopsies. Diagn. Microbiol. Infect. Dis. **13**:473–479.
12. **Jackson, D. P., F. A. Lewis, G. R. Taylor, A. W. Boylston, and P. Quirke.** 1990. Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. J. Clin. Pathol. **43**:499–504.
13. **Jones, D. M., and J. Eldridge.** 1987. Gastric campylobacter-like organisms (GCLO) from man (*C. pyloridis*) compared with GCLO strains from the pig, baboon and ferret, p. 44. *In* B.

Kaijser and E. Falsen (ed.), *Campylobacter* IV: Proceedings of the Fourth International Workshop on *Campylobacter* Infection. University of Göteborg, Göteborg, Sweden.
14. **Kogan, S. C., M. Doherty, and J. Gitschier.** 1987. An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences. N. Engl. J. Med. **317**:985–990.
15. **Lau, P. P., B. DeBrunner-Vossbrinck, B. Dunn, K. Miotto, M. T. MacDonnell, D. M. Rollins, C. J. Pillidge, R. B. Hespell, R. R. Colwell, M. L. Sogin, and G. E. Fox.** 1987. Phylogenetic diversity and position of the genus *Campylobacter*. Syst. Appl. Microbiol. **9**:231–238.
16. **Lee, A., S. L. Hazell, J. O'Rourke, and S. Kouprach.** 1988. Isolation of a spiral-shaped bacterium from the cat stomach. Infect. Immun. **56**:2843–2850.
17. **Lo, Y.-M. D., W. Z. Mehal, and K. A. Fleming.** 1989. *In vitro* amplification of hepatitis B virus sequences from liver tumour DNA and from paraffin wax embedded tissues using the polymerase chain reaction. J. Clin. Pathol. **42**:840–846.
18. **Malloy, D. C., R. K. Nauman, and H. Paxton.** 1990. Detection of *Borrelia burgdorferi* using the polymerase chain reaction. J. Clin. Microbiol. **28**:1089–1093.
19. **Marshall, B. J., J. R. Warren, G. J. Francis, S. R. Langton, C. S. Goodwin, and E. D. Blincow.** 1987. Rapid urease test in the management of *Campylobacter pyloridis*-associated gastritis. Am. J. Gastroenterol. **82**:200–210.
20. **McNulty, C. A. M., J. C. Dent, A. Curry, J. S. Uff, G. A. Ford, M. W. L. Gear, and S. P. Wilkinson.** 1989. New spiral bacterium in gastric mucosa. J. Clin. Pathol. **42**:585–591.
21. **Morotomi, M., S. Hoshina, P. Green, H. C. Neu, P. LoGerfo, I. Watanabe, M. Mutai, and I. B. Weinstein.** 1989. Oligonucleotide probe for detection and identification of *Campylobacter pylori*. J. Clin. Microbiol. **27**:2652–2655.
22. **Newell, D. G., M. J. Hudson, and A. Baskerville.** 1988. Isolation of a gastric campylobacter-like organism from the stomachs of four rhesus monkeys, and identification as *Campylobacter pylori*. J. Med. Microbiol. **27**:41–44.
23. **Newell, D. G., and A. R. Stacey.** 1989. The serology of *Campylobacter pylori* infections, p. 74–82. *In* B. J. Rathbone and R. V. Heatley (ed.), *Campylobacter pylori* and gastroduodenal disease. Blackwell Scientific Press, Oxford.
24. **Olive, D. M.** 1989. Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with a thermostable DNA polymerase. J. Clin. Microbiol. **27**:261–265.
25. **Pääbo, S., R. G. Higuchi, and A. C. Wilson.** 1989. Ancient DNA and the polymerase chain reaction. The emerging field of molecular archaeology. J. Biol. Chem. **264**:9709–9712.
26. **Paster, B. J., and F. E. Dewhirst.** 1988. Phylogeny of campylobacters, wolinellas, *Bacteroides gracilis*, and *Bacteroides ureolyticus* by 16S ribosomal ribonucleic acid sequencing. Int. J. Syst. Bacteriol. **38**:56–62.
27. **Paster, B. J., A. Lee, J. G. Fox, F. E. Dewhirst, L. A. Tordoff, G. J. Fraser, J. L. O'Rourke, N. S. Taylor, and R. Ferrero.** 1991. Phylogeny of *Helicobacter felis* sp. nov., *Helicobacter mustelae*, and related bacteria. Int. J. Syst. Bacteriol. **41**:31–38.
28. **Porter-Jordan, K., E. I. Rosenberg, J. F. Keiser, J. D. Gross, A. M. Ross, S. Nasim, and C. T. Garrett.** 1990. Nested polymerase chain reaction for the detection of cytomegalovirus overcomes false positives caused by contamination with fragmented DNA. J. Med. Virol. **30**:85–91.
29. **Rauws, E. A. J., and G. N. J. Tytgat.** 1990. Cure of duodenal ulcer associated with eradication of *Helicobacter pylori*. Lancet **i**:1233–1235.
30. **Romaniuk, P. J., B. Zoltowska, T. J. Trust, D. J. Lane, G. J. Olsen, N. R. Pace, and D. A. Stahl.** 1987. *Campylobacter pylori*, the spiral bacterium associated with human gastritis, is not a true *Campylobacter* sp. J. Bacteriol. **169**:2137–2141.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
32. **Steer, H. W.** 1989. Ultrastructure of *Campylobacter pylori in vivo*, p. 146–154. *In* B. J. Rathbone, and R. V. Heatley (ed.), *Campylobacter pylori* and gastroduodenal disease. Blackwell Scientific Press, Oxford.
33. **Thompson, L. M., III, R. M. Smibert, J. L. Johnson, and N. R. Krieg.** 1988. Phylogenetic study of the genus *Campylobacter*. Int. J. Syst. Bacteriol. **38**:190–200.
34. **Tompkins, D. S., J. I. Wyatt, B. J. Rathbone, and A. P. West.** 1988. The characterisation and pathological significance of gastric campylobacter-like organisms in the ferret: a model for chronic gastritis? Epidemiol. Infect. **101**:269–278.
35. **Valentine, J. L., R. R. Arthur, H. L. T. Mobley, and J. D. Dick.** 1991. Detection of *Helicobacter pylori* by using the polymerase chain reaction. J. Clin. Microbiol. **29**:689–695.
36. **Vary, P. H., P. R. Andersen, E. Green, J. Hermon-Taylor, and J. J. McFadden.** 1990. Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. J. Clin. Microbiol. **28**:933–937.
37. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature (London) **345**:63–65.
38. **Weil, J., and G. D. Bell.** 1989. Detection of *Campylobacter pylori* by the ^{14}C-breath test, p. 83–93. *In* B. J. Rathbone, and R. V. Heatley (ed.), *Campylobacter pylori* and gastroduodenal disease. Blackwell Scientific Press, Oxford.
39. **Wilson, K.** 1987. Preparation of genomic DNA from bacteria, p. 2.4.1–2.4.2. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl (ed.), Current protocols in molecular biology 1987–88. Greene Publishing Associates and Wiley-Interscience, New York.
40. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**:221–271.
41. **Wright, D. K., and M. M. Manos.** 1990. Sample preparation from paraffin-embedded tissues, p. 153–158. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, San Diego.