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# Reverse Transcription and Polymerase Chain Reaction Amplification of rRNA for Detection of *Helicobacter* Species

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**Sequence data on *Helicobacter pylori* 16S rRNA were used to select two 22-base oligonucleotide primers for use in a polymerase chain reaction (PCR) for detection of *H. pylori*. *H. pylori* cells were treated with lysis buffer, boiled, and chloroform extracted. Reverse transcription of rRNA was followed by PCR amplification (RT-PCR) of the synthesized cDNA and 16S rRNA gene. The amplified PCR products were analyzed by agarose gel electrophoresis and Southern blotting. Using ethidium bromide-stained agarose gels, we were able to detect the expected 500-bp DNA fragment from as few as two *H. pylori* organisms per reaction. The specificity of the RT-PCR assay was tested with 27 clinical isolates and related reference strains; although the number of bacterial cells used per reaction was $10^5$-fold greater than the number of *H. pylori* organisms used, amplification was detected only with bacteria in the same genus, *H. cinaedi* and *H. mustelae*. Ten *H. pylori* organisms per biopsy specimen were detected on agarose gels when organisms were added to samples prepared from a processed colon biopsy sample. RT-PCR results were consistent with urea breath test and culture results in 14 of 15 gastric biopsy specimens; the specificity was 100%. RT-PCR of rRNA from *H. pylori* increased the sensitivity of pathogen detection at least 25- to 50-fold compared with that of previous PCR assays. This low level of detection by RT-PCR assay may prove to be well suited for verifying eradication following therapy.**

*Helicobacter pylori* is an important cause of gastritis in humans and plays a role in the pathogenesis of peptic ulcer disease (8). It is relatively easy to identify the presence of an active *H. pylori* infection by histological staining or culture of gastric mucosal biopsy specimens or indirectly by biopsy specimen urease tests or urea breath tests (UBTs) (1, 2, 5). However, none of these tests is ideal for identification of recent incompletely treated infections nor for searching for *H. pylori* in environmental samples or feces. Several nucleic acid techniques have been developed to increase the sensitivity of detection of *H. pylori* (13, 23, 25); the sensitivity of detection was further improved by amplification of the target DNA by polymerase chain reaction (PCR) followed by oligoprobe or amplified PCR product probe detection of the reaction products (3, 10, 11, 21). Because some regions of rRNA are highly conserved while others are highly variable, rRNA sequences have proved to be extremely useful for studies of bacterial taxonomy (9, 15, 19). The high copy number of rRNA per bacterial cell also allows enhanced detection sensitivity several thousandfold greater than that of DNA-RNA hybridization techniques (24). Recently, an oligonucleotide probe complementary to *H. pylori* 16S rRNA in a DNA-RNA dot blot assay was shown not to cross hybridize with a panel of related and unrelated bacteria obtained from gastric biopsy cultures (4, 11, 13). In this study we evaluated this putative *H. pylori*-specific probe by using its complementary sequence as one of two primers in RNA reverse transcription and cDNA PCR amplification (RT-PCR) for the detection of *H. pylori*. We have developed a sensitive and rapid technique for the detection of *Helicobacter* species in gastric biopsy specimens that is based on the amplification of nucleic acid sequences from 16S rRNA. The specificity was assessed by hybridization to the amplified RT-PCR probes.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The following reference bacteria were used in this study: *Arcobacter butzleri* CCUG 10373, *Bacteroides ureolyticus* CCUG 9510, *Campylobacter coli* CCUG 11283, *Campylobacter curvus* CCUG 13146 and CCUG 11644, *Campylobacter hyointestinalis* CCUG 14169, *Campylobacter jejuni* subsp. *doylei* CCUG 24567, *C. jejuni* subsp. *jejuni* CCUG 11284, *Campylobacter rectus* CCUG 11640, *Campylobacter upsaliensis* CCUG 14913, *Helicobacter cinaedi* CCUG 18819, *Helicobacter mustelae* CCUG 25715, and *H. pylori* RD26. Twelve reference strains were obtained from the Culture Collection, University of Gothenburg Department of Clinical Bacteriology, Gothenburg, Sweden, and *H. pylori* RD26 was a gift from D. G. Evans, Veterans Affairs Medical Center, Houston, Tex. The reference strains were grown on 7% horse blood agar plates under microaerobic conditions at 37°C for 72 h as previously described (1). In addition, 15 clinical isolates (*Achromobacter xylosoxidans*, *Campylobacter fetus*, *C. jejuni*, *Citrobacter diversus*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Shigella flexneri*, *Staphylococcus aureus*, *Vibrio cholerae*, and *Vibrio parahaemolyticus*) were kindly provided by Jill Clarridge (Clinical Microbiology Laboratory, Veterans Affairs Medical Center, Houston, Tex.) and were isolated and identified by standard microbiologic procedures (12).

**Preparation of nucleic acid extracts for PCR.** Chromosomal DNA from the reference strain *H. pylori* RD26 was prepared from freshly harvested bacterial cells that were washed

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twice with STE buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The washed cell pellet was suspended in 0.5 ml of the same buffer containing lysozyme (egg white, grade 1; Sigma Chemical Co., St. Louis, Mo.) at 3.5 mg/ml. After incubation for 30 min at 37°C, proteinase K was added to a final concentration of 0.1 mg/ml and the incubation was continued for an additional 30 min. Sodium dodecyl sulfate (SDS) then was added to a final concentration of 1% (wt/vol), and the mixture was incubated for 2 h at 37°C. The lysate was treated with RNase A (GIBCO BRL, Gaithersburg, Md.) at a final concentration of 100 μg/ml and incubated further for 60 min. The DNA was extracted twice with an equal volume of a mixture of phenol–chloroform-isoamyl alcohol (24:1, vol/vol). After another extraction with chloroform-isoamyl alcohol, the DNA was ethanol precipitated and the pellet was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA concentration was determined spectrophotometrically at 260 nm.

To investigate whether *H. pylori* could be detected without an extensive purification process, crude nucleic acid extracts of whole organisms also were prepared from all the reference strains, including *H. pylori* RD26, and the clinical isolates listed above. Tenfold serial dilutions of fresh bacterial cells (from suspensions adjusted to an optical density of 1) of each bacterial strain were prepared in lysis buffer consisting of 10 mM Tris-HCl, 1 mM EDTA [pH 8.0], 10 mM NaCl, and 1% Triton X-100. After thorough mixing, 200 μl of each diluted lysate (containing $4 \times 10^5$ to 4 organisms) was boiled for 30 min and the nucleic acids were extracted with an equal volume of chloroform-isoamyl alcohol in Phase Lock Gel tubes (5′→3′ Inc., Paoli, Pa.) according to the manufacturer's instructions. The aqueous phase containing the nucleic acid was stored at −20°C until use in PCR.

To determine the possible inhibitory effect of proteinase K on PCR, a similar set of 10-fold serial dilutions of *H. pylori* RD26 whole organisms was prepared by digestion with proteinase K at a final concentration of 0.4 mg/ml for 1 h at 55°C before the boiling step. In addition, two sets of 10-fold serial dilutions containing 10 ng to 1 pg of genomic DNA in 200 μl of lysis buffer were prepared with and without proteinase K. All the diluted samples were boiled, chloroform extracted, and stored as described above.

**Clinical samples.** To investigate detection of *H. pylori* in clinical specimens, initially three biopsy samples from patients with *H. pylori* culture-positive gastritis were obtained from the prepyloric area, immediately placed in carrier media (1), and stored at −70°C. After a gentle washing in TE buffer, each biopsy specimen was transferred into an Eppendorf tube and homogenized with a disposable pestle homogenizer (Kontes, Vineland, N.J.). The homogenate then was resuspended in 200 μl of lysis buffer, boiled for 30 min, and chloroform extracted as described above. The aqueous layer containing nucleic acid templates of each sample was stored at −20°C until use.

To ensure that the primers did not hybridize to human nucleic acid sequences, a colon biopsy specimen from a patient with ulcerative colitis was processed in parallel with the biopsy specimens described above. To exclude possible inhibitory effects on PCR by substances present in the processed biopsy specimen, we added crude *H. pylori* RD26 nucleic acid prepared from 1,000, 200, and 10 organisms to nucleic acid samples from the colon biopsy specimens. To test the applicability and efficiency of this assay on clinical specimens, 15 coded frozen biopsy samples were randomly picked, processed, and stored as described above. These samples were obtained from patients with duodenal ulcers and gastric ulcers and from healthy volunteers (see Table 1). These patients were successfully or unsuccessfully treated or undergoing therapy at the time of these biopsies and were monitored by culture and UBT for *H. pylori*.

**PCR primers.** Sequence data of *H. pylori* 16S rRNA (17, 19) were used to select and custom synthesize (Genosys Biotechnologies Inc., The Woodlands, Tex.) two 22-base oligonucleotide primers designated HP1 (5′-TGGCAAT CAGCGTCAGGTAATG-3′) and HP2 (5′-GCTAAGAGAT CAGCCTATGTCC-3′). Because the complete 16S rRNA sequence of *H. pylori* with the precise base numbers was not available to us, the location of these primers was assessed by using the 16S rRNA nucleotide sequence of *C. jejuni* from GenBank. HP1 is complementary to the region at nucleotides 719 to 740, and HP2 is complementary to the species-specific region located at nucleotides 219 to 240 of *H. pylori* 16S rRNA (4, 11, 13). The expected product of amplification of the target cDNA produced by reverse transcription with these primers was as expected: approximately 500 bp in length.

**RT-PCR.** Amplification of *H. pylori* DNA sequences was carried out in a total volume of 100-μl containing PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.0], 2.5 mM $MgCl_2$ [$MgCl_2$ concentration was optimized by titration experiments], and 3% [vol/vol] glycerol), 200 μM (each) deoxynucleotides (dATP, dCTP, dTTP, and dGTP), 180 ng of each oligonucleotide primer, 2.5 U of AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and a 10-μl volume of DNA sample (diluted pure DNA). Prior to the addition of DNA templates, each reaction mixture was overlaid with 50 μl of light mineral oil. The reaction mixes were subjected to an initial denaturation step at 94°C for 5 min and then 39 cycles of amplification performed in a programmable thermal controller (MJ Research, Watertown, Pa.) as follows: 1-min denaturation at 94°C, 1-min annealing at 55°C, and 3-min primer extension at 72°C. After the 39th cycle, the extension step was continued for another 10 min at 72°C.

To investigate the sensitivity of *H. pylori* detection by RT-PCR assay, the production of cDNA was performed in a total volume of 30 μl containing PCR buffer with the above concentrations of each deoxynucleotide, 180 ng of HP1 primer, 40 U of RNasin (Promega Biotec, Madison, Wis.), 100 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL), and a 10-μl volume of nucleic acid sample (crude extracts). The transcription reaction mixes were incubated for 60 min at 37°C. PCR amplification of the synthesized cDNA was then carried out by adding another 70 μl of PCR buffer containing 180 ng of HP2 primer and 2.5 U of AmpliTaq polymerase. The tubes were subjected to the same thermal profile as described above with 39 cycles of amplification.

To control possible contamination, all plasticware was dedicated for exclusive use in the preparation of PCR and was sterile and UV irradiated daily. Aerosol barrier pipet tips and dedicated pipettors were used for the preparation of reaction mixes. All reaction buffers and solutions not obtained from commercial sources were sterile and prepared with water obtained from a Milli-Q water system (Millipore Corp., Bedford, Mass.). Reaction mixes were prepared under sterile conditions in a laminar flow hood that is dedicated only for tissue culture medium preparation and located in a room separate from that used for DNA preparations and examination of PCR products. No PCR products were taken into the PCR preparation area. Negative and positive controls were prepared for every experiment.

**Preparation of DNA probes.** Amplified target sequence

from the reference strain (*H. pylori* RD26) was used as the probe in Southern blot hybridizations. After amplification, reaction mixes were electrophoresed through 1% agarose gels containing 0.5 μg of ethidium bromide per ml and the PCR DNA products were visualized by UV fluorescence. The bands of interest were recovered by electrophoresis onto DEAE-cellulose membranes (NA45; Schleicher & Schuell, Keene, N.H.) (7). Fragments were eluted from the membranes in high-salt buffer (50 mM Tris-Cl [pH 8.0], 1.0 M sodium chloride, 10 mM EDTA [pH 8.0]) and extracted with 2 volumes of *n*-butanol and then with an equal volume of phenol–chloroform-isoamyl alcohol (1:1, vol/vol). The DNA fragments were ethanol precipitated, dissolved in water, and stored at −20°C. Concentrations of eluted nucleic acids were determined spectrophotometrically at 260 nm. The DNA probes were prepared by the hexanucleotide priming technique with digoxigenin-11-dUPT by using the Genius labelling kit by following the manufacturer's instructions (Boehringer Mannheim, Indianapolis, Ind.). The labelling reaction mixtures were incubated for 3 h, after which additional reagents (half the suggested amounts per reaction) were added and the incubation period was extended for 18 h. After ethanol precipitation, the labelled DNA probes were suspended and diluted in TE solution containing 0.1% SDS to a final concentration of 5 ng/ml and stored at −20°C until use.

**Southern blots and hybridization analysis.** For gel and Southern blot analysis, 20-μl aliquots of the amplified samples were analyzed by electrophoresis on agarose gels containing 0.5 μg of ethidium bromide per ml. PCR DNA products migrating at approximately 500 bp were visualized and photographed over UV light. The amplified fragments were transferred onto nylon membranes (Hybond-N+; Amersham, Arlington Heights, Ill.) by the alkaline blotting procedure using 0.4 N sodium hydroxide (16). After overnight transfer, the membranes were washed briefly in 2× SSC (1× SSC consists of 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) and air dried.

Hybridizations with digoxigenin-labelled probes were performed by using the Genius kit as directed by the manufacturer. Briefly, membranes were prehybridized in a blocking solution consisting of 2× SSC, 0.1% *N*-lauroylsarcosine, 0.5% blocking reagent (supplied in the Genius kit), and 0.1% SDS for 5 h at 65°C. After denaturation at 100°C for 10 min, the DNA probe was added to the prehybridized membranes at 25 ng/ml together with fresh blocking solution. Hybridization proceeded at 65°C for 16 to 20 h. Membranes were washed twice (5 min each) at room temperature in 2× SSC–0.1% SDS and then twice (30 min each) at 65°C in 0.1× SSC–0.1% SDS. The membranes were then blocked and labelled hybridized probes were detected with the Genius detection kit as directed by the manufacturer.

## RESULTS

**RT-PCR assay of *H. pylori* nucleic acids.** The oligonucleotide sequence that is complementary to HP2 primer has been shown in hybridization experiments to be a highly species-specific probe for the detection of *H. pylori* (4, 11, 13). In a similar dot blot hybridization experiment, this specificity was confirmed when the same oligonucleotide probe bound specifically to clinical isolates representing 10 different *H. pylori* strains, obtained from patients with asymptomatic gastritis or *H. pylori*-associated duodenal ulcers (including the reference strain, RD26), and did not hybridize with *C. jejuni*, *C. fetus*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa*

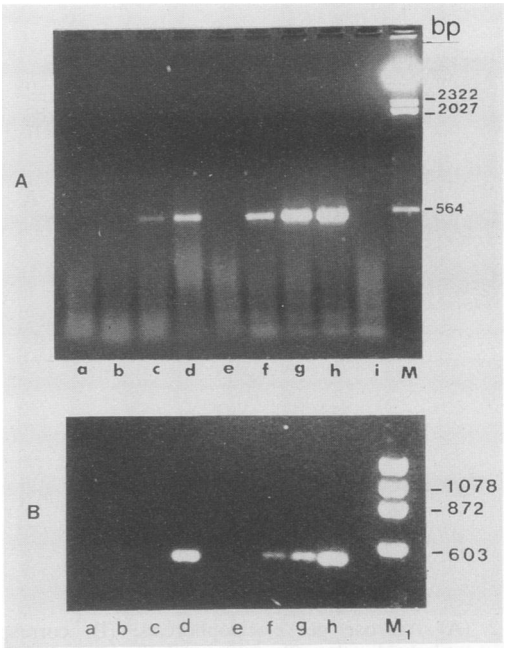


FIG. 1. Sensitivity of RT-PCR and PCR detection of the *H. pylori* RD26 16S rRNA gene and the inhibitory effect of proteinase K on amplification reactions. Analysis of amplified products is shown on 1% agarose gels. (A) RT-PCR. Boiled crude DNA extracts of 10-fold serial dilutions of whole organisms prepared in lysis buffer with (lanes a to d) and without (lanes e to h) proteinase K. The number of bacteria per reaction was as follows: 1, lanes a and e; 2, lanes b and f; 20, lanes c and g; 200, lanes d and h; and 0, lane i. (B) PCR. Boiled extracts of 10-fold serial dilutions of chromosomal DNA prepared in lysis buffer with (lanes a to d) and without (lanes e to h) proteinase K. The amount of DNA per sample was as follows: 50 fg, lanes a and e; 0.5 pg, lanes b and f; 5 pg, lanes c and g; and 500 pg, lanes d and h. Lane M, *Hin*dIII-digested lambda DNA; lane M1, *Hae*III-digested ϕX174 DNA. For details, see Materials and Methods.

(data not shown). The detection sensitivity of these assays was at least $10^3$ to $10^4$ bacterial cells in all *H. pylori* samples tested.

To improve the sensitivity of detection, PCR and RT-PCR assays were employed. HP1 and HP2 primers were tested in these assays with diluted whole-cell crude extracts and purified DNA prepared from reference strain RD26 by boiling in lysis buffer with and without proteinase K, respectively. Addition of 10 μl of crude DNA extract prepared from the appropriate dilution of RT-PCR mixture was amplified to produce the expected band of 500 bp from as few as two bacterial cells per reaction (Fig. 1A, lane f). However, when proteinase K was added to the lysis buffer of either the diluted crude extracts (Fig. 1A) or genomic DNA preparations (Fig. 1B), the detection limit was reduced 10- and 100-fold, respectively, as shown in Fig. 1 (lanes a to d with and lanes e to h without proteinase K). After proteinase K tested negative for DNase activity, its inhibitory effect on these PCR and RT-PCR assays was confirmed when the same experiments were repeated twice with identical results. These observations indicate that nucleic acid extraction by the boiling method is very efficient even with small numbers of *H. pylori* organisms and that the primers are sufficient to detect small numbers of bacteria. In addition, as proteinase K produced an inhibitory effect on PCR, it was not used in subsequent experiments.

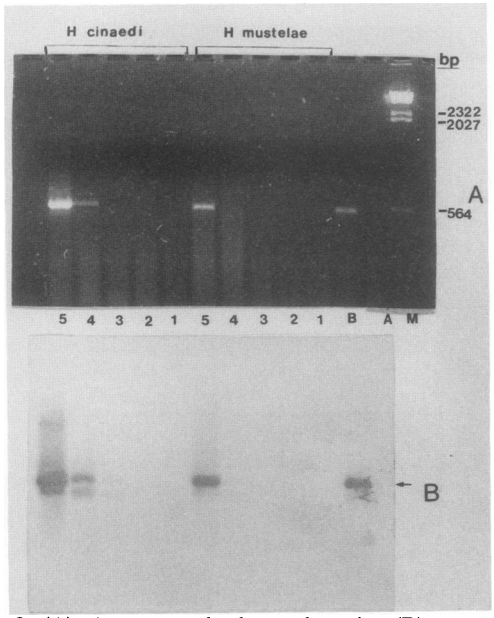


FIG. 2. (A) Agarose gel electrophoresis; (B) corresponding Southern blot hybridization. Tenfold serial dilutions of each organism (*H. cinaedi* and *H. mustelae*) were prepared without proteinase K, boiled, processed, and analyzed as described in the legend to Fig. 1. The numbers of organisms per reaction were 2, 20, 200, $2 \times 10^3$, and $2 \times 10^6$ in lanes 1 to 5, respectively. Lane A, lysis buffer only (negative control); lane B, 20 *H. pylori* organisms; lane M, *Hin*dIII-digested lambda DNA.

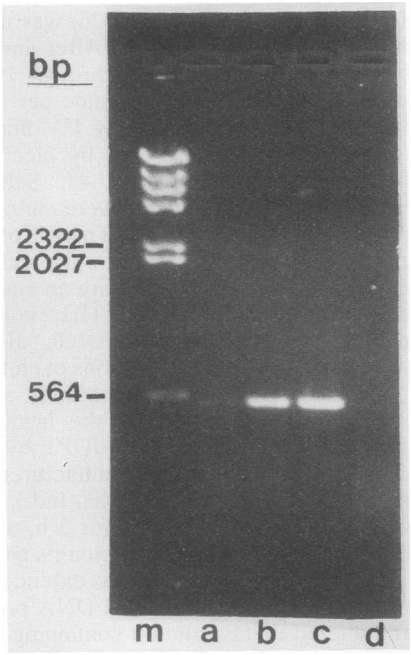


FIG. 3. RT-PCR assay performed on colon biopsy specimen homogenate samples spiked with *H. pylori* nucleic acid. A colon biopsy specimen from an ulcerative colitis patient was homogenized and boiled, and the lysate samples containing human nucleic acids were spiked with diluted crude extracts of *H. pylori* as described in Materials and Methods. The amplified products were analyzed on an agarose gel. The number of bacteria spiked into each sample was as follows: 10, lane a; 200, lane b; $10^3$, lane c; and 0, lane d. Lane m, molecular size marker.

In order to demonstrate the specificity of this RT-PCR, HP1 and HP2 primers were also tested with diluted aliquots in lysis buffer of each sample (10 μl) containing crude DNA templates prepared from 20 *H. pylori* RD26 and $2 \times 10^6$ bacterial cells of other related and unrelated reference strains and clinical isolates that are listed in Materials and Methods. Although the number of bacterial cells from other species was around $10^5$-fold higher than the number of *H. pylori* organisms, no detectable amplification was found (either in agarose gel or in blots) with crude extracts prepared from all the clinical isolates listed in Materials and Methods, with the exception of two species that are in the same genus, *H. mustelae* and *H. cinaedi*, as shown in Fig. 2. However, the sensitivity of detection of these *Helicobacter* species per reaction varied from at least $2 \times 10^3$ organisms for *H. mustelae* to $2 \times 10^2$ organisms for *H. cinaedi* by probe hybridization (Fig. 2). To confirm these observations and to rule out any possible contaminations, we repeated these experiments with fresh batches of cultures of the same strains, and identical results were attained. The size of the amplified fragment from the *Helicobacter* species that were tested was, as expected, 500 bp.

It should be emphasized that the conditions of RT-PCR assay were optimized experimentally. No false positives resulting from contamination or carryover was observed. These results confirm the specificity and sensitivity of this RT-PCR assay for the detection of *Helicobacter* 16S rRNA and its gene.

**Detection of *H. pylori* in gastrointestinal tissues.** As an initial step to explore its application to clinical samples, we have used this RT-PCR assay on three gastric biopsy specimen homogenates obtained from culture-positive patients with *H. pylori* gastritis. A control colon biopsy specimen homogenate obtained from a patient with ulcerative colitis was also used. HP1 and HP2 primers did not hybridize to human nucleic acids, as demonstrated in Fig. 3, lane d; no amplification was observed when crude extract from the colon biopsy specimen was used as a template. Furthermore, there was no major inhibitory effect from the biopsy specimen components, as can be seen by the amplification of *H. pylori* RD26 nucleic acid-spiked colon biopsy samples (Fig. 3, lanes a to c); the amplified PCR products from 10, 200, and 1,000 bacterial cells were detected in an agarose gel.

When crude nucleic acid extracts prepared from two of the above culture-positive gastric biopsy specimen homogenates were used as templates in RT-PCR, the expected amplified fragment from each sample was also observed in the agarose gel (Fig. 4, lanes A and B). In addition, a significant decrease in the detection sensitivity was observed when crude nucleic acid extract from the third biopsy specimen was amplified without the reverse transcription step (data not shown). In order to examine the efficiency of this assay, 15 additional gastric biopsy specimen homogenates were randomly picked, processed, and RT-PCR assayed. The expected amplified fragments from five positive biopsy specimens were observed in the agarose gel (Fig. 5A, lanes 1, 5, 9, 10, and 12). The number of positive biopsy specimens was increased to eight when *H. pylori* PCR probe was used (Fig. 5B, lanes 6, 11, and 13). These results were compared with results of the UBTs and cultures that were performed for the same patients; results for 14 of 15 (93.3%) specimens were consistent with the comparative results (Table 1). To confirm the results of negative biopsy specimen 2, another biopsy specimen from the same patient (obtained 2 months after

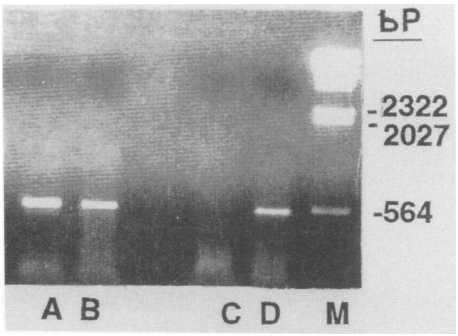


FIG. 4. RT-PCR performed on gastric biopsy specimens. Two biopsy specimens obtained from patients with *H. pylori*-associated chronic gastritis of the antrum were processed as described in the legend to Fig. 3. The lysate samples containing human nucleic acids were amplified by RT-PCR. The amplified products were analyzed on an agarose gel. Lane A, biopsy sample 1; lane B, biopsy sample 2; lane C, lysis buffer only (negative control); lane D, crude extract prepared from 250 *H. pylori* RD26 bacteria (positive control); lane M, molecular size markers.

biopsy specimen 2 was extracted) was RT-PCR tested and gave the expected product on Southern blot. The false-negative biopsy specimen 2 result by RT-PCR was attributed to the patchy colonization nature of the infection (26), emphasizing the importance of testing at least two biopsy specimens per patient (14). The patchy nature of the infection is further illustrated by the results with biopsy specimen 13 (Table 1). This patient was known to be infected, and the culture was negative; the UBT and RT-PCR were positive. Studies are under way to evaluate RT-PCR for its ability to reliably test and monitor the eradication of *H. pylori* in these patients.

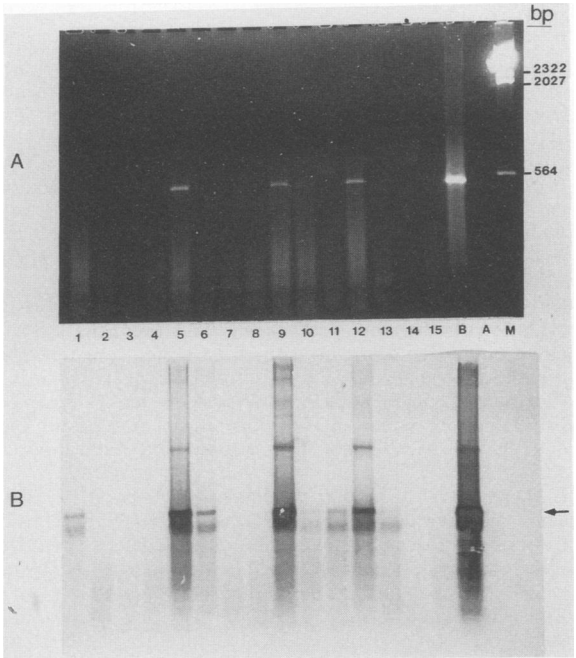


FIG. 5. Application of RT-PCR amplification to gastric biopsy samples. (A) Agarose gel electrophoresis; (B) corresponding Southern blot hybridization. Crude lysate extracts from 15 gastric biopsy samples were used for amplification. Lane A, lysis buffer only (negative control); lane B, 20 *H. pylori* organisms per reaction; lane M, molecular size markers.

TABLE 1. Comparison of RT-PCR and various assays for detecting *H. pylori* in gastric biopsy samples

| Biopsy specimen no. | Patient age (yr) | Clinical diagnosis[a] | Result of[b]: | | |
|---|---|---|---|---|---|
| | | | UBT | Culture | RT-PCR |
| 1 | 50 | GU | NT | + | + |
| 2 | 58 | DU | + | + | −[c] |
| 3 | 73 | DU | − | − | − |
| 4 | 61 | DU | − | NT | − |
| 5 | 70 | GU | + | + | + |
| 6 | 51 | DU | + | + | + |
| 7 | 54 | DU | − | − | − |
| 8 | 70 | GU | − | − | − |
| 9 | 37 | DU | + | + | + |
| 10 | 36 | DU | + | + | + |
| 11 | 33 | DU | + | + | + |
| 12 | 64 | DU | + | + | + |
| 13 | 65 | DU | + | − | + |
| 14 | 28 | HV | − | − | − |
| 15 | 55 | DU | − | − | − |

[a] DU, duodenal ulcer; GU, gastric ulcer; HV, healthy volunteer.
[b] NT, not tested.
[c] Another biopsy specimen from this patient was obtained and tested positive by RT-PCR (see text for details).

## DISCUSSION

Because of the clinical importance of *H. pylori* infection and the lack of a rapid and sensitive technique for its detection in clinical samples, several diagnostic procedures based on nucleic acid technology have been developed (13, 23, 25). The application of DNA amplification for the detection of microorganisms makes it possible to increase the concentration of a specific gene sequence $>10^6$-fold (18). Recently, amplification of DNA sequences by PCR has been shown to enhance the sensitivity and rapidity of hybridization procedures for the detection of *H. pylori* in clinical samples (3, 10, 11, 21).

The large number of 16S rRNAs in a bacterial cell holds a promise of increasing the target DNA copies (templates) if reverse transcription is combined with the power of the PCR amplification technique and hence enhancing the sensitivity of bacterial detection. In this study, we have used a specific oligonucleotide probe derived from the *H. pylori* 16S rRNA nucleotide sequence as one of the primers to develop and optimize conditions for a specific RT-PCR assay and assessed its applicability for the detection of very low numbers of *H. pylori* organisms. Our RT-PCR assay reliably identified the amplified fragment, approximately 500 bp, from as few as two bacteria on an ethidium bromide-stained agarose gel (Fig. 1A). Because of the high concentration of rRNA per bacterial cell, the amplified products were visualized with enhanced sensitivity when Southern hybridization was employed (data not shown). PCR amplification of the *H. pylori* 16S rRNA gene with specific primers has been already described by Hoshina et al. (11). However, no information was given for the sensitivity of their PCR assay. When the *H. pylori* 16S rRNA gene was used as a template in a DNA-based PCR assay, a detectable band at 500 bp was observed from an amplified reaction mixture containing an estimated 50 to 100 organisms per reaction (data not shown). These results are in agreement with the recently published PCR assays that were based on DNA sequences for the

detection of *H. pylori* (3, 10, 21). However, these limits of detection are at least 25- to 50-fold lower in sensitivity compared with the agarose gel results of our RT-PCR, as shown in Fig. 1A, lane f. In gastric biopsies, this loss of sensitivity was obvious when a biopsy specimen was analyzed by the conventional DNA-based PCR method (data not shown).

The specificity of our RT-PCR assay was assessed by testing 27 related and unrelated clinical isolates and reference strains. Despite the high number of these organisms ($10^5$-fold more than *H. pylori*) used per reaction, only the two bacteria in the same genus that were tested, *H. cinaedi* and *H. mustelae*, showed detectable and same-size amplified products that cross hybridized with the *H. pylori* RT-PCR probe. These results were confirmed when new batch cultures of these two *Helicobacter* species were obtained from the Culture Collection, University of Gothensburg, and tested several times, at annealing temperatures of 55°C as well as 60°C, with identical results. However, it was surprising to find that the sensitivity of detection varied among these three different *Helicobacter* species: 2, $2 \times 10^2$, and $2 \times 10^3$ for *H. pylori*, *H. cinaedi*, and *H. mustelae*, respectively (Fig. 1A, lane f, and Fig. 2B, lanes 3 and 4). Previous studies by different investigators evaluated the specificity of their PCR assays using *H. mustelae* as the only other *Helicobacter* species (3, 10, 21). Although specific assays were reported by these investigators, quantitative analysis of the related and unrelated bacteria that were included in their studies was not given in comparison to a known number *H. pylori* organisms. Hoshina et al. (11) also used *H. cinaedi* but did not report the quantity used in the reaction compared to *H. pylori*. Thus, it would be difficult to compare the specificity of previous *H. pylori* PCR assays to that of our RT-PCR assay. In addition, our results emphasize the crucial importance, when evaluating the specificity of a PCR assay, not only to include very closely related bacterial species, namely, species in the same genus, but also to evaluate them quantitatively.

*H. cinaedi* was isolated from the blood and rectal swabs of homosexual males with intestinal symptoms, while *H. mustelae* was isolated from stomachs of ferrets with chronic gastritis (6, 20). The observed amplification of *H. cinaedi* and *H. mustelae* strongly supports the results of recent phylogenetic studies (15, 19). These species not only are related to each other but also have many genotypic and phenotypic characteristics in common (22). Although *H. pylori* species-specific PCR assays and now this RT-PCR assay (that appears to be genus specific) have been developed, other newly classified species in the genus *Helicobacter* need to be examined quantitatively before claiming that a probe is species or genus specific. Whether these organisms play a role in the pathogenesis of chronic active gastritis also needs to be further investigated.

We have demonstrated that this RT-PCR combined with Southern hybridization is useful for rapid and accurate identification of intestinal *Helicobacter* species. Although not species specific, our assay demonstrated a good correlation (93.3 to 100% specificity) with UBT and culture results. These results are similar to those recently reported by Valentine et al. (21) and Clayton et al. (3), who used DNA-based PCR to detect *H. pylori* in gastric aspirates and biopsy specimens, respectively. However, radioactive probes were used in both studies, a procedure which is not suitable to routine clinical laboratory use. Considering the sensitivity of detection (two organisms per reaction), our assay may be suitable to serve as an alternative or a useful adjunct to existing procedures in the diagnostic laboratory. A future application is to search for other vegetative and nonculturable coccoid forms of *Helicobacter* species which may be present in gingival scrapings, oral secretions, or feces, thus allowing determination of the source and route of infection of the organism and enabling the epidemiology of this organism to be determined in more detail.

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