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Rapid Detection of *Helicobacter pylori* in Gastric Biopsy Material by Polymerase Chain Reaction

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By using primers based on the sequence of a species-specific antigen of *Helicobacter pylori* (P. O'Toole, S. M. Logan, M. Kostrzynska, T. Wadström, and T. J. Trust, J. Bacteriol. 173:505–513, 1991), a protocol was established for detection of this microorganism in gastric biopsy samples by the polymerase chain reaction (PCR). A single primer pair was used to specifically amplify a 298-bp sequence in a rapid two-step PCR. The primers exhibited the same specificity in PCR as that which we reported for the species-specific gene probe on which they were based. The sensitivity of the method was 20 copies of the target sequence, or 70 bacterial cells, under the lysis conditions used for patient-derived material. When amplification was performed for a saturating number of cycles, visual examination of ethidium bromide-stained gels successfully detected all samples subsequently judged to be positive by Southern hybridization of the gel with a probe specific for the PCR product. The bacterium could be detected in gastric biopsy samples from patients with various gastric diseases, including samples from which the bacterium could not be cultured. Only 9 of 19 patients who tested positive by PCR of gastric biopsy material were positive when a saliva sample was analyzed. Protocols for sample handling which minimized the risk of contamination while maximizing the sensitivity of the reaction were established. The results support a role for PCR in the rapid identification of *H. pylori* in clinical samples.

Helicobacter pylori (formerly *Campylobacter pylori* [7]) is a curved or spiral gram-negative microaerophilic bacterium which was first isolated from a human gastric biopsy specimen in 1983 (27). Since this first isolation, it has become apparent that this organism may be one of the most common bacterial pathogens of humans. Epidemiological evidence has shown that *H. pylori* colonizes the upper gastrointestinal tract of more than one in two individuals during their life spans, and in many of these people, the organism is associated with disease of the gastrointestinal tract (9, 10, 15). Indeed, *H. pylori* appears to be causally associated with active and chronic gastritis as well as peptic and duodenal ulcers (1, 2, 5, 9, 15), and it may also be associated with the development of carcinoma of the stomach (12, 14).

A variety of diagnostic procedures are used for the identification of *H. pylori* in clinical samples (reviewed by Westblom [28]). No single test is optimal because of length of time required to perform the test, lack of sensitivity, or irreproducibility. Culture and biochemical identification is specific and has an advantage in that antimicrobial therapy can be planned on the basis of the organism's susceptibility, but it has the associated disadvantages of lack of sensitivity and long incubation periods. Its success in identifying the bacterium is directly dependent on the efficient and expedient handling of clinical samples, normally gastric biopsy material. Like other approaches, such as direct microscopy and histological staining, it also requires performance of a gastroscopy, with its accompanying costs.

The potent urease produced by *H. pylori* (21) formed the basis for a simple diagnostic procedure (17) which has subsequently been modified extensively (3). Most of these urease tests rely on the existence of preformed urease in gastric biopsy specimens, obviating the need for growth. An elegant modification, which has the advantage of being noninvasive, is the so-called urea breath test, which monitors the release of labeled CO_2 following ingestion of isotopically labeled urea (8, 10). The original version of the test with ^{13}C offers a stable isotope which can be used in children and pregnant women, but the detection equipment is more expensive than that which is necessary for detection of ^{14}C. Use of the latter isotope is cheaper and more sensitive, but it does entail long-term exposure, admittedly at a low level, to this long-lived isotope.

H. pylori elicits both local and systemic immune responses, resulting in elevated titers of serum immunoglobulins G and A. Accordingly, a range of serological tests based on antigen preparations of various origins and efficacies have been described (reviewed by Dick [3]; see also Newell and Stacey [19]). The antigens used in many are ill defined or completely unknown. Sensitivities and specificities of 98.7 and 100%, respectively, are reported. Previous studies in our clinic have recorded 90% seropositivity in patients with positive gastric culture for *H. pylori* when a serology based on an acid glycine extract of bacterial cells was used (11).

DNA-based approaches to the detection of *H. pylori* have included the use of radiolabeled and antigenically labeled chromosomal fragments, in situ hybridization, and hybridization with an oligonucleotide based on 16S rRNA sequence (18, 26, 29). More recently, a protocol for detection by the polymerase chain reaction (PCR) was described; the PCR was based on DNA sequence analysis of a cryptic fragment cloned from the *H. pylori* genome (25). This study demonstrated the feasibility of using PCR for the rapid, sensitive, and specific detection of *H. pylori*.

We describe here the establishment of an experimental protocol for detection by PCR of *H. pylori* in gastric biopsy samples. The assay was based on the DNA sequence of a species-specific protein antigen which was present in all

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strains of *H. pylori* tested and whose coding sequence did not hybridize to the DNAs of numerous enteric bacteria (20). The results support the feasibility of using primers based on the sequence that encodes this antigen for detection systems that use DNA amplification by PCR. The sensitivity, speed, and potential for automation should make this an alternative, or at least a useful adjunct, to existing procedures in the diagnostic laboratory.

MATERIALS AND METHODS

Bacterial strains. The sources of the 13 strains of *H. pylori*, the 3 *Helicobacter mustelae* strains, the 19 *Campylobacter* sp. strains, and the 9 gram-negative enteric organisms used in this study have already been described (20). Culture and storage conditions were as documented in that report (20).

Chemicals. Analytical-grade reagents were from Boehringer (Mannheim, Germany) or Sigma (St. Louis, Mo.).

Biopsy samples. Gastric biopsy samples were taken from patients (ages, 20 to 86 years) who were referred to the endoscopy unit of the Department of Surgery, Hässleholm Hospital, Hässleholm, Sweden, for evaluation (see Table 1). All gastric biopsy samples were examined histopathologically at the Department of Pathology, Malmö General Hospital, Malmö, Sweden, by a single pathologist. Excised tissue samples were transferred to Stuart's transport medium (6) and were processed in the laboratory within 24 h.

Sample processing. Upon arrival from the clinic, tissue samples and the accompanying transport medium were divided in half. One of the halves was processed for routine culture of *H. pylori*. After considerable preliminary investigation for maximizing sensitivity and minimizing the risk of contamination, the following procedure was adapted for preparing the material in the remaining half for PCR. The sample was transferred to a microcentrifuge tube with a disposable plastic pipette, and insoluble material was harvested by centrifugation for 5 min in a microcentrifuge. The supernatant was decanted, and 300 μl of extraction buffer (20 mM Tris HCl [pH 8.0], 0.5% Tween 20) was added. The pellet was resuspended by vortexing, and proteinase K was added to a final concentration of 0.5 mg/ml. After incubation for 1 h at 55°C, the proteinase K was inactivated by heating the solution to 98°C for 10 min. A 10-μl aliquot was removed at this stage; this represented preparation A (see Table 1). The main sample was extracted once by vortexing with an equal volume of phenol-chloroform, and after 1 min of centrifugation in the microcentrifuge, the aqueous layer was transferred to a new tube. From this, a 10-μl aliquot was withdrawn (preparation B). The DNA present in the deproteinized sample was precipitated by the addition of sodium acetate to 0.3 M (from a 3 M solution [pH 5.2]) and 2.5 volumes of ethanol; this was followed by a 30-min incubation at −70°C. The DNA was recovered by centrifugation for 15 min, the residual alcohol was removed, and the pellet was resuspended in 30 μl of water (preparation C).

Saliva treatment. To the saliva sample (typically 0.5 ml in a 10-ml plastic tube) was added 2 volumes of Sputolysin (Behring Diagnostics, La Jolla, Calif.), and the tube was shaken gently for 20 min at room temperature. Insoluble material was harvested by centrifugation for 5 min in a microcentrifuge, and the pellet was resuspended in 300 μl of extraction buffer. The sample was then processed as described above for gastric biopsy samples beginning with the addition of proteinase K.

PCR primers. A number of candidate primer pairs were initially synthesized (Scandinavian Gene Synthesis, Köping, Sweden) on the basis of partial DNA sequence derived for a species-specific antigen of *H. pylori* (20). Following preliminary trials, the following primer pair was selected for further study: 5′-(TGGCGTGTCTATTGACAGCGAGC)-3′ and 5′-(CCTGCTGGGCATACTTCACCATG)-3′. These oligonucleotides were designated primers 3 and 4, respectively, and are identical to residues 474 to 496 and 776 to 754 of the published sequence, respectively.

PCR amplification. Reactions were performed in a volume of 100 μl in 1.5-ml microcentrifuge tubes. An Intelligent Heating Block (Hybaid, Teddington, England) was used for thermal cycling. Reactions comprised the four deoxynucleotides (Pharmacia, Sweden), each at 100 μM, and 0.1 μM (each) primers 3 and 4 in a standard PCR incubation buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin). The sample volume did not normally exceed 3 μl. The mixture was overlaid with 100 μl of light mineral oil (Sigma) and denatured at 98°C for 10 min. The temperature was reduced to 68°C, and 1 U of AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) in a volume of 2 μl was added without removal of the tube from the heating block. After 1 min at 68°C and 30 s at 92°C, an additional 37 cycles were performed, each consisting of annealing at 68°C for 1 min and denaturation at 92°C for 30 s. A final additional six cycles were performed, in which a 2-min elongation step at 72°C was interposed between the annealing and denaturation steps described above. A total of 44 cycles were therefore executed.

One aspect of establishing an assay was to determine the possible effect of inhibitory impurities on the PCR. Accordingly, all clinical samples which were initially negative by PCR were subjected to a second PCR in which positive control DNA was added and checked for its amplifiability.

The amplified product was analyzed by electrophoresis in 3% agarose gels (NuSieve GTG agarose; FMC Products, Rockland, Maine). Standard procedures were used for the analysis of chromosomal and plasmid DNAs and Southern transfer (23). In Southern blot hybridization experiments, the PCR product amplified from 4 ng of genomic DNA from *H. pylori* 915 was used. The fragment was isolated from an agarose gel by electroelution (23). This DNA probe was nonisotopically labeled by incorporation of digoxigenin-dUTP by the procedures included in the commercial kit (Boehringer), and hybridization conditions were as recommended by the vendor.

RESULTS

Optimization of PCR parameters. The concentrations of each of the components of the PCR and the thermocycling parameters were each optimized by using 1 ng of *H. pylori* genomic DNA as the target. To simulate actual working conditions, this positive control DNA was processed as described above for gastric biopsy samples before the PCR. A $MgCl_2$ concentration of 2.5 mM gave the best yield; above this concentration there was a decline in yield. Under no conditions, however, did aberrant bands appear. The concentrations of the other components indicated above yielded the combination which best reconciled economical running costs with maximum specificity and sensitivity. In preliminary experiments, we used a standard three-step PCR with a 1-min extension step, but further investigation showed that comparable yields were obtained with a two-step PCR for the bulk of the cycling program, with considerable savings in the total run time. Many standard three-step PCR protocols include prolonged extension times in the final few cycles,

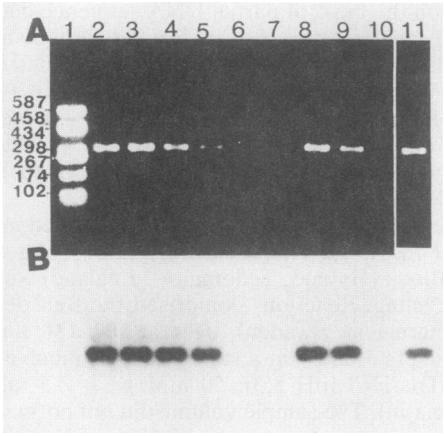

FIG. 1. Agarose gel electrophoresis (A) and corresponding Southern blot hybridization (B) of the PCR product from cloned target sequence (p26K) and *H. pylori* 915 cells. Serial dilutions were amplified in the standard PCR assay, separated by electrophoresis in 3% agarose, and then probed with the amplified 298-bp fragment. Lane 1, pUC18 DNA digested with *Hae*III to generate linear standards (indicated on the left in base pairs); lane 2, positive control amplification of 1 ng of genomic DNA of *H. pylori* 915; lanes 3 to 7, dilutions of plasmid p26K containing 2,000, 200, 20, 2, and 0 copies of the plasmid, respectively; lanes 8 to 10, 700, 70, and 7 cells of *H. pylori* 915, respectively; lane 11, representative positive PCR assay performed on a gastric biopsy sample. The results shown for serial dilutions are typical for three independent experiments.

and the superior yield obtained is presumably due to the more effective use of a limiting polymerase concentration as the product increases. We obtained better overall yields and sensitivity by including six final cycles that incorporated an extension step.

Specificity and sensitivity of the PCR. Previous work has shown that when the entire cloned fragment of 870 bp, which encodes the antigen with an M_r of 26,000, was used as a probe against numerous *H. pylori* strains, *H. mustelae* isolates, *Campylobacter* spp., and various gram-negative enteric organisms, only *H. pylori* strains hybridized (20). The same panel of strains was tested in the PCR described here, with the result that only *H. pylori* target cells gave rise to an amplification product. Under reduced or elevated $MgCl_2$ concentrations or reduced annealing temperatures, some of the non-*H. pylori* species yielded low amounts of product of various sizes, presumably because of nonspecific primer annealing under suboptimal conditions. Human DNA from a variety of cell types was also negative for the presence of the target sequence in the PCR assay.

The sensitivity of detection was investigated by performing the PCR on dilutions of a recombinant plasmid, p26K (20), which contained the cloned fragment. A 10-μl aliquot from each reaction was electrophoresed on a 3% agarose gel, which was then analyzed both by staining and Southern hybridization. The minimum number of target copies detectable by probing the PCR product was 20, an endpoint which was also detectable by direct photography of the stained gel (Fig. 1). Since lysis of the bacterial cells that are present in clinical samples could not be assumed to be 100%, the endpoint was also tested for dilutions of a standard bacterial suspension (enumerated by direct microscopy). These dilutions were processed by the extraction procedure used for clinical samples and were then subjected to PCR. By this procedure, 70 bacterial cells were detectable (Fig. 1).

TABLE 1. Bacterial culture, PCR assay, serological data, and clinical diagnosis of patients with various gastric disorders

Patient no.	Age (yr)	Bacterial culture	PCR assay result for the following samples and preparations[a]:						Serology value (RAA[b])	Diagnosis
			Gastric biopsy			Saliva				
			A	B	C	A	B	C		
3	75	+	+	+	−	+	−	−	84	NUD[c]
14	35	+	+	+	+	−	+	+	117	Duodenal ulcer
32	82	+	+	+	+	+	−	−	10	NUD
33	49	−	−	−	−	−	−	−	38	Gastric ulcer
69	29	+	+	+	−	−	−	−	70	NUD
70	45	−	−	−	−	−	−	−	18	NUD
71	47	−	+	+	−	−	−	−	23	Duodenal ulcer
72	20	−	−	−	−	−	−	−	8	NUD
73	42	−	−	−	−	−	−	−	50	NUD
74	21	−	−	−	−	−	−	−	5	NUD
80	77	+	+	+	−	−	−	−	20	Esophagitis
81	72	−	−	−	−	−	−	−	15	VBR[d]
82	36	+	+	+	−	−	−	−	74	NUD
84	68	−	+	+	+	−	−	−	158	Gastric ulcer
85	74	−	−	−	−	−	−	−	35	Esophagitis
86	58	+	+	+	+	−	−	−	42	Duodenal ulcer
87	50	−	−	−	−	−	−	−	36	NUD
88	64	−	+	−	−	−	−	−	34	NUD
89	64	+	+	+	−	−	−	−	36	Esophagitis
90	69	+	+	+	+	−	−	−	36	Esophagitis
97	59	+	+	+	−	−	−	−	141	Duodenal ulcer
213	36	−	+	+	+	+	+	+	3	NUD
214	86	+	+	+	+	+	+	+	13	Gastric ulcer
215	74	+	+	+	+	+	+	−	21	Gastric ulcer
218	47	+	+	+	+	−	+	+	81	Gastric ulcer
219	67	+	+	+	+	−	+	+	74	NUD
231	59	+	+	+	+	−	+	+	70	NUD

[a] Positivity was confirmed in three separate experiments.
[b] Relative antibody activity in a standardized ELISA, as defined in the text.
[c] NUD, Nonulcer dyspepsia.
[d] VBR, Ventricular Billroth I surgery (removal of part of stomach in a patient with excessive obesity).

Detection of *H. pylori* in gastric biopsy material by PCR. As a pilot study of the applicability of PCR to the detection of *H. pylori* in gastric biopsy material and saliva, the standardized assay was applied to samples from 27 patients presenting with a variety of gastric disorders. All patients had antral gastritis confirmed by histopathology according to standard criteria (4).

Each biopsy sample was tested by PCR at three stages of purity represented by preparations A, B, and C, as described in Materials and Methods. The results of this analysis, together with serological statuses and clinical diagnoses, are presented in Table 1. Serology values are expressed as relative antibody activity by reference to an enzyme-linked immunosorbent assay (ELISA) run in parallel with human gamma globulin. A cutoff for positivity is arbitrarily set at 40 relative antibody activity units, and values between 20 and 40 are considered intermediate. The diagnoses tabulated are based on consideration of the clinical picture by a single gastrologist.

The data indicate that PCR performed on gastric biopsy samples identified all samples which were also culture positive for *H. pylori* and all but one sample identifiable by serology using an arbitrary cutoff value. PCR detected the presence of *H. pylori* in four samples which were culture negative. For all gastric biopsy samples, the earliest of the

three preparations tested (preparation A) was already PCR positive. Only nine saliva samples were PCR positive, all of which were from patients whose gastric biopsy samples tested positive by PCR. In four of these nine cases, detection was facilitated by purification of the target DNA by phenol extraction.

DISCUSSION

Because of the fastidious nature of the organism and the lengthy culture times required, a rapid and sensitive system for the detection of *H. pylori* based on nucleic acid has been an attractive alternative. Earlier systems based on hybridization with genomic probes and oligonucleotides have been followed by a recent description of PCR-based detection (25). Valentine and coworkers (25) based their primers on the sequence of a cryptic fragment cloned from the *H. pylori* chromosome. Their PCR assay was shown to be specific and had sensitivities very similar to those we described here (approximately 100 bacterial cells). Both methods represent a significant improvement on the most sensitive of the hybridization-based protocols, namely, use of an oligonucleotide based on the 16S rRNA sequence (18). Our PCR system should be shorter in total running time than that described by Valentine and coworkers (25), because we adapted a two-step thermal profile. It is likely, however, that their protocol could also work adequately without the annealing step.

The data presented here, which were derived from a relatively small number of samples, are the results of a pilot study that preceded a long-term project encompassing PCR, serology, and endoscopy in monitoring a larger patient population. One of our goals at this stage was to document the possible effects of the degree of contamination of the target DNA by inhibitory materials on the outcome of the PCR. The results show that at least for biopsy samples, sensitivity is not improved by deproteinization and alcohol precipitation. On the contrary, the insidious DNA loss associated with these procedures is probably the explanation for the negativity in the PCR assay of seven samples at the preparation C stage (these samples were all positive at the preparation A and B stages). There was a single example of a sample which was positive only at the first stage. It is probable that these samples contained very low numbers of bacteria and that the loss of target DNA during cleaning procedures was sufficient to push this number below the lower threshold for detectability. In all cases in which a patient sample proved to be negative in the PCR assay (eight such samples), positive control DNA was added to the sample and was shown to be amplifiable, ruling out the possibility that negativity was due to excessive impurities inhibiting the PCR.

It is obvious from the data obtained with gastric biopsy samples that PCR is more sensitive than either culture or serology for detecting the presence of *H. pylori*. Of 19 samples that were positive in the PCR assay, only 15 of these were culture positive for *H. pylori*, and only 10 were positive by serology. Six of the remaining serology-negative samples had intermediate titers but were PCR positive, perhaps indicating that the arbitrary limits set for serology cutoff points could bias against low-grade infection. Three other samples from patients with intermediate serology titers were negative by PCR, again underlining the possible difficulty of deciding from serological data whether or not the patient is infected. It is possible that in this type of patient the ELISA detects a long-lived antibody response to *H. pylori* antigens well after an active infection has ceased.

The failure of culture to detect the bacterium in four biopsy samples which were positive by the PCR assay could be due to loss of viability in transport, but rigorous procedures were instigated to prevent this. Alternatively, the PCR assay may have detected the controversial "resting form" of the bacterium (16), a proposed phase into which the vegetative form of the bacterium might differentiate under conditions of environmental stress. Regardless of this possibility, the PCR assay can detect the target DNA whether the bacteria are viable or not, provided that gross breakdown of nucleic acids has not occurred. This innate sensitivity could be important if rigorous transport procedures from surgery to the diagnostic laboratory are difficult to implement.

The same extreme sensitivity can cause problems of contamination, and as for other PCR systems, we exercised caution in separating post-PCR material from sample handling areas and reagent preparation stations. Newly developed procedures for tagging the post-PCR product with enzymatically degradable nucleotide derivatives (22) should become routine when PCR is applied for diagnostic purposes. During our initial studies of patient-derived material, we suspected cross-contamination of negative controls from positive samples. We adapted the sample preparation procedure described above, with emphasis on the use of a minimum number of preparative steps, disposable pipettes, and positive displacement pipettes, to successfully solve this problem. All series of PCR assays routinely contained negative control vials, dispersed throughout the range of samples, to detect contamination, and all were negative by the protocol described here.

Only nine of the saliva samples tested were positive in the PCR assay, and there was no obvious correlation with serological or clinical data. We were prompted to examine saliva because of reports of cultivation of *H. pylori* from dental plaque (13, 24), although we did not attempt to cultivate the organism from saliva in this study. It is possible that random environmental factors exert an effect on the presence or absence of bacterial cells in saliva, such as the frequency of reflux or the time that the sample was taken with respect to the patient's latest meal. Our findings so far are too preliminary to rule out the detectability of *H. pylori* in saliva by PCR. We are evaluating saliva, feces, and gastric aspirates by PCR.

The aim of this study was to show that the PCR technique can be successfully applied for the detection of *H. pylori* in gastric biopsy material. The patients from which these samples were taken covered a spectrum of ages and had a variety of gastric disorders, and we do not seek to infer that there is a definite link between the presence of the bacterium and disease in these patients. Examination of such relationships is ongoing. As part of long-term clinical studies, the PCR assay has the advantage of detecting low numbers of bacteria after successful or unsuccessful therapy or prior to relapse and will, we hope, permit the evaluation of the efficacy of various antimicrobial regimens.

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