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Evaluation of a Novel Heminested PCR Assay Based on the Phosphoglucosamine Mutase Gene for Detection of *Helicobacter pylori* in Saliva and Dental Plaque

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A novel heminested PCR protocol was developed for the specific detection of *Helicobacter pylori* at low copy numbers. A set of primers specific for the phosphoglucosamine mutase gene (*glmM*) of *H. pylori* produced a 765-bp fragment that was used as template for the heminested primer pair delineating a 496-bp fragment. By using agarose gel electrophoresis for detection of the heminested PCR-amplified products, amplification of *H. pylori* genomic DNA was achieved at concentrations as low as 0.1 pg, equivalent to 5×10^2 bacteria. A study was subsequently undertaken to evaluate the heminested PCR for detection of *H. pylori* in dental plaque and saliva. Specimens collected from 58 individuals were cultured, and PCR was subsequently performed on the oral cultures. Identification of *H. pylori* in the same series of saliva and dental plaque specimens was carried out with PCR using a primer pair specific for the *H. pylori* urease B gene and by the heminested PCR assay. The identity of the amplified products was confirmed by DNA sequencing. Our results demonstrate that the heminested PCR assay was specific for detection of *H. pylori*, yielding no false-positive results, and that *H. pylori* had a low prevalence (approximately 3%) in specimens obtained from the oral cavity.

Helicobacter pylori is a microaerophilic, spiral-shaped, motile gram-negative bacterium and is strongly associated with gastroduodenal disease, including chronic active gastritis, peptic and duodenal ulcer disease, and gastric cancer (reviewed in references 16 and 22). Although *H. pylori* infection is widespread throughout the world (21), the mode of transmission, the natural history, and other aspects of the epidemiology of *H. pylori* infection are still unclear. Reported observations support a person-to-person mode of transmission via the fecal-oral, oral-oral, and gastro-oral routes (18, 43).

Several methods for detecting *H. pylori* are used at present. Most of these diagnostic tests are performed on gastric biopsy samples, and the bacterium can be identified in these specimens by a urea hydrolysis test, staining techniques, and culturing (14, 38). Both the urease test and microscopic analysis of stained biopsy smears, however, suffer from low sensitivity (10), and conventional plate counting is tedious and time-consuming and usually underestimates the number of these organisms (7). Since invasive methods are expensive, less-invasive methods such as serologic examination of blood and the urea breath test are becoming more popular (47). However, positive results by blood serology do not necessarily allow the delineation of a current active *H. pylori* infection from a past *H. pylori* infection, while urea breath tests require expensive, specialized equipment and reagents (5).

Based on the difficulty of culturing *H. pylori* from sites other than the gastric mucosa (20) and the need for noninvasive diagnostic methods, interest has grown in the use of molecular techniques for detection of this species. The use of gene-specific probes has been described for the detection of *H. pylori* in biopsy specimens (25, 44), and progress has been made by use of PCR, which provides a specific and highly sensitive means of detecting microbial pathogens in clinical material. PCR assays have detected *H. pylori* DNA in fresh gastric biopsy specimens (7, 12, 17, 23, 30), in feces (33, 45), in saliva (23, 30), and in dental plaque (3, 7, 8, 11, 41). The detection of *H. pylori* in dental plaque and saliva suggests that the oral cavity may be an important reservoir for this organism. Many studies have been published recently that support and contradict this theory (2, 4, 7, 8, 11, 24, 31, 34, 35, 37, 39, 41). Most of these studies have used PCR assays on dental plaque and saliva specimens and reported a 0 to 97% incidence of positive samples. As these results are not in agreement with the prevalence of *H. pylori* infection in the stomach, it is questionable as to whether they represent the real prevalence of this microorganism in the human oral cavity or are artifacts of the methods applied. It has been speculated that the conflicting PCR results obtained concerning *H. pylori* presence in the oral cavity could in part be due to the use of PCR primers of differing sensitivities and specificities as well as the use of samples from different patient groups (40). Thus, the hypothesis that the oral cavity may be a permanent reservoir of viable *H. pylori* still remains a controversial issue.

In this study, we report the development of a heminested PCR assay based on the amplification of a specific internal region of the phosphoglucosamine mutase gene (*glmM*) of *H. pylori*. We furthermore evaluated the heminested PCR assay for the detection of *H. pylori* in saliva and dental plaque by comparison with an established PCR assay.

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MATERIALS AND METHODS

Subjects, sampling, and bacterial isolates. Oral specimens, consisting of dental plaque and sulcus fluid, were collected with sterile toothpicks and filter paper, respectively, from 58 randomly selected, clinically healthy volunteers attending the Gastroenterology Unit at Pretoria Academic Hospital, Pretoria, South Africa. Informed consent was obtained from each individual and the ethics committee of the University of Pretoria, South Africa, approved the use of these specimens for research. All oral specimens were inoculated onto brain heart infusion agar plates (Merck, Darmstadt, Germany) supplemented with 5% (vol/vol) sheep blood and cultured as described previously (46). Presumptive *H. pylori* isolates were identified based on the presence of urease and catalase activity.

DNA extraction. Suspensions of each bacterial strain were prepared in sterile double-distilled water (200 μl) by inoculation of a single bacterial colony from agar plates with a standard loop. The bacterial cells were subsequently subjected to a freeze-thaw lysis procedure. Briefly, bacterial suspensions were frozen at −70°C for 8 min followed by thawing at 40°C for 3 min. These cycles were repeated five times with intermittent vortexing, after which the cellular debris was removed by centrifugation at 10,000 × *g* for 1 min. The supernatant fluid was recovered and 5 μl of DNA was used as template in the PCRs.

Selection and synthesis of primers. Oligonucleotide primers HPU50 (5′-GA ACATGACTACACCAT-3′) and HPU25 (5′-TGGTTTGAGGGCGAATC-3′) have been described previously (13). These primers yield a 933-bp PCR product of the *H. pylori* urease B gene. Primers glmMF (5′-CGCGAGCCACAACCCT TTTGAAG-3′) and glmMR (5′-CGCGCTCACTTGCAAAGCGCACAC-3′) as well as an internal primer, glmMI (5′-GCTTATCCCCATGCACGATATTC-3′), were designed based on the *glmM* gene sequence reported by Labigne et al. (29). The first PCR step, performed with primers glmMF and glmMR, amplified a 765-bp region of the *H. pylori glmM* gene, while the size of the final PCR product obtained with primers glmMF and glmMI was 496 bp. The primers were synthesized by MWG-Biotech (Ebersberg, Germany) with automatic DNA synthesizers.

Primer specificity. Oligonucleotide primers glmMF, glmMR, and glmMI were tested for amplification specificity using DNA extracted from a panel of bacterial strains, as indicated in Table 1.

PCR amplification. (i) Heminested PCR. The template DNA was added to 50 μl of a reaction mixture containing 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5% [vol/vol] Triton X-100), 1.5 mM $MgCl_2$, 200 μM concentrations of each deoxynucleoside triphosphate, 50 pmol of each primer (glmMF and glmMR), and 1 U of *Taq* DNA polymerase (Promega, Madison, Wis.). Ten cycles at 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min were performed in a Perkin-Elmer Thermocycler 2400. After PCR, 1 μl of the final product was transferred to the second-round reaction mixture and reamplified for 20 cycles with the glmMF and glmMI pair of primers under the conditions described above, with a final extension step at 72°C for 5 min. Positive control reactions consisted of *H. pylori* genomic DNA from type strain ATCC 700392, while negative controls contained double-distilled water instead of sample DNA.

(ii) Single-step PCRs. The PCRs with primers HPU50 and HPU25 were performed as described previously (13). For primer pair glmMF and glmMR, the reactions were subjected to 30 cycles of amplification under the cycle conditions described above. The amplicons were analyzed with 2% (wt/vol) agarose gels, and the sizes of the amplicons were estimated by comparison with 100-bp DNA size markers (Promega).

Sequence determination of PCR amplicons. PCR amplicons were purified from agarose gels using the QIAEXII DNA extraction kit (QIAGEN, Hilden, Germany). DNA sequencing was performed using the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Applied Biosystems, Foster City, Calif.) with an Applied Biosystems 377 DNA sequencer. Amplified DNA fragments were sequenced in both orientations using 10 pmol of the appropriate oligonucleotide primers (HPU50, HPU25, glmLF, or glmMR). The sequence data were analyzed with the Sequencing Analysis 3.1 and Sequencing Navigator 1.0.1 programs included in the ABI PRISM software package (Perkin-Elmer, Applied Biosystems). Sequence comparison was subsequently carried out using the BLAST 2.1 software program and the GenBank database.

TABLE 1. Bacterial strains[a] used for testing *glmM* primer specificity

Bacterial species
Arcobacter butzleri clinical isolate
A. cryaerophila clinical isolate
Actinomyces naeslundii NCTC 10301
Bacillus cereus clinical isolate
Campylobacter coli clinical isolate
Campylobacter concisus EF 13144
Campylobacter fetus clinical isolate
Campylobacter hyointestinalis NCTC 10608
Campylobacter jejuni clinical isolate
Campylobacter lari clinical isolate
Campylobacter mucosalis NCTC 11000
Campylobacter upsaliensis clinical isolate
Enterobacter aerogenes ATCC 49469
Enterococcus faecalis ATCC 49474
Escherichia coli ATCC 13086
Klebsiella pneumoniae ATCC 49472
Lactobacillus paracasei clinical isolate
Proteus mirabilis ATCC 12453
Prevotella intermedia ATCC 25611
Salmonella typhimurium ATCC 49469
Shigella sonnei ATCC8574
Shigella flexneri CCRC 10772
Streptococcus cricetus clinical isolate
Streptococcus ferus clinical isolate
Streptococcus macacae clinical isolate
Streptococcus mutans ATCC 10449
Streptococcus mutans ATCC 25175
Streptococcus salivarius clinical isolate
Streptococcus sobrinus clinical isolate
Vibrio cholerae NCTC 5941
Helicobacter cinaedi NCTC 11611
H. fennelliae NCTC 11612
H. pylori ATCC 700392
H. pylori clinical isolate 102.90
H. pylori clinical isolate 114.90
H. pylori clinical isolate 201.90
H. pylori clinical isolate 206.90 Ig
H. pylori clinical isolate 206.90 sm
H. pylori clinical isolate 220.90
H. pylori clinical isolate 228.90
H. pylori clinical isolate 237.90
H. pylori clinical isolate 242.90 li
H. pylori clinical isolate 242.90 da
H. pylori clinical isolate 513.90

[a] Clinical isolates were obtained from the Red Cross Children's Hospital, Rondebosch, Cape Town, South Africa; South African Institute for Medical Research, Johannesburg, South Africa; Witwatersrand University Medical School, Johannesburg, South Africa; and the Council for Scientific and Industrial Research, Pretoria, South Africa.

RESULTS

Specificity of the primers. The specificity of the oligonucleotide primer pair HPU50 and HPU25 had been previously demonstrated (13). To investigate the specificity of the glmMF-glmMR PCR, DNA samples of all strains listed in Table 1 were subjected to 30 cycles of amplification. Only the *H. pylori* strains yielded an intense band of the expected length corresponding to 765 bp. With all other strains tested, no PCR product was detectable. To confirm and to validate amplicon integrity, we performed sequencing reactions on aliquots of the purified 765-bp amplicons generated in PCR.

To investigate the specificity of the heminested PCR, the bacterial extracts were subjected to a 10-cycle PCR amplification with primers glmMF and glmMR, and 1 μl of the first-round PCR product was subjected to another 20 cycles of amplification with oligonucleotides glmMF and the nested primer, glmMI. Only the *H. pylori* strains yielded an amplicon of the expected size (approximately 496 bp) on agarose gel electrophoresis. No similar amplification product was observed from microorganisms other than *H. pylori* (Table 1 and Fig. 1).

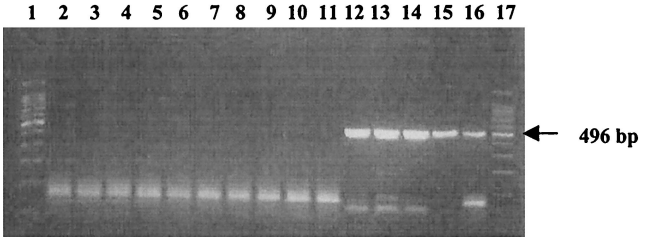

FIG. 1. Specific detection of *H. pylori* by heminested PCR. Shown is agarose gel electrophoresis of amplicons obtained from cells of *Arcobacter butzleri*, *Campylobacter conciscus* EF 13144, *Campylobacter hyointestinalis* NCTC 10608, *Campylobacter mucosalis* NCTC 1100, *Lactobacillus paracasei*, *Streptococcus cricetus*, *Streptococcus salivarius*, *Streptococcus sobrinus*, *Helicobacter cinaedi* NCTC 11611, *Helicobacter fennelliae* NCTC 11612, *H. pylori* ATCC 700392, *H. pylori* isolate 114.90, *H. pylori* isolate 206.90 sm, *H. pylori* isolate 228.90, and *H. pylori* isolate 242.90 Da (lanes 3 to 16, respectively). The arrow indicates the 496-bp amplified fragments obtained only with *H. pylori* (lanes 12 to 16). Lane 2, negative control (no DNA template). Lanes 1 and 17, 100-bp ladder as a molecular size standard (Promega).

However, amplification of a smaller 100-bp fragment from *H. pylori* isolates was occasionally observed (Fig. 1, lane 13). Because this fragment was only amplified in the presence of *H. pylori* but was not observed when other bacterial strains were used, we attribute its presence to amplification of this fragment from these organisms rather than amplification of DNA from any other bacteria. We therefore concluded that both the single-step and heminested PCR assays could be used to specifically detect *H. pylori* DNA sequences.

Detection limits of the assay. The detection limits of the PCR assays were investigated by preparing serial dilutions of genomic DNA from *H. pylori* type strain ATCC 700392 in sterile double-distilled water. An aliquot (1 μl) of each dilution was subjected to PCR amplification, and the products were visualized on 2% agarose gels stained with ethidium bromide. With the single primer pair, glmMF and glmMR, the 765-bp fragment could be visualized in ethidium bromide-stained gels in reaction mixtures that contained 10 pg of total chromosomal DNA. This level of detection is equivalent to the detection of 5×10^4 *H. pylori* cells. To attain a lower detection limit with simultaneous confirmation of the reaction product, the samples were subjected to the heminested PCR assay. The application of this procedure rendered possible the detection of 0.1 pg of DNA (5×10^2 cells) without loss of specificity. Thus, detection levels were increased 100-fold in the heminested PCR assays.

***H. pylori* in saliva and dental plaque samples.** Oral specimens from 58 individuals were inoculated onto brain heart infusion agar, but only 8 samples yielded colonies with a morphology resembling that of *H. pylori*. These cultures were purified, and a limited number of biochemical tests (microaerophilic growth, production of catalase and urease) were performed. No further bacteriological identification of oral cultures was performed.

From this selected group, five isolates were negative in both PCR assays and were thus considered to be non-*Helicobacter* spp. Only three samples (two saliva and one dental plaque) yielded the expected 933-bp amplification product by PCR using primers HPU50 and HPU25. However, of these three samples, only two were positive by heminested PCR. One of the two saliva samples that tested positive by HPU50-HPU25 PCR was negative in the heminested PCR assay (Fig. 2). On each occasion when PCR was carried out, negative controls were always negative and positive controls were always positive, thereby excluding the possibility of contamination.

Confirmation of product specificity. To confirm that the amplicons obtained from the PCR-positive samples were indeed derived from *H. pylori*-specific DNA, DNA sequencing was performed on the amplified products following their purification from the agarose gels. The DNA sequence data of the HPU50-HPU25 PCR-amplified DNA fragments demonstrated that only two of the amplicons originated from the *H. pylori* urease B gene sequence (GenBank accession no. M60398). These results were in agreement with the positive results obtained by heminested PCR with the corresponding samples. Sequence analysis of the amplicon from the saliva sample that was positive by the HPU50-HPU25 PCR but negative in the heminested PCR showed that it was 99% identical to a genomic clone from *Staphylococcus epidermidis* (GenBank accession no. AF270031).

These results were confirmed in a second independent experiment by PCR amplification of a clinical isolate as well as a type strain of *S. epidermidis* (ATCC 12228). PCR using HPU50 and HPU25 primers yielded amplicons in both of the samples corresponding to the expected size (933 bp) for amplification of *H. pylori*-specific DNA. In contrast, no amplification products were observed in the heminested PCR assay (results not shown). Thus, these results served to further confirm the specificity of the heminested PCR assay for *H. pylori*.

DISCUSSION

In recent years, diagnostic laboratories have been concerned with reducing the time required for diagnosis of *H. pylori* infections. Different methods based on molecular biology techniques have therefore been developed for identification of *H.*

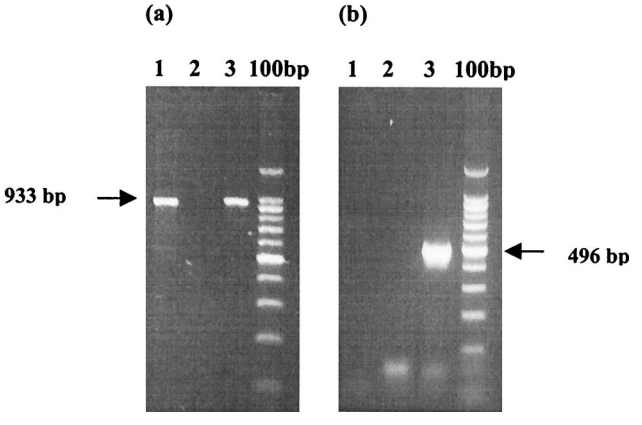

FIG. 2. PCR with primers HPU50 and HPU25 (a) and heminested PCR (b) from a saliva sample, indicating nonspecific amplification following analysis by agarose gel electrophoresis of the amplified products. Lanes: 1, saliva specimen; 2, negative control (no DNA template); 3, *H. pylori* positive control. 100bp, 100-bp ladder as a molecular size standard (Promega). The sizes of the respective amplicons are indicated by arrows.

pylori from clinical specimens, with those based on DNA amplification being the most rapid and sensitive. Nevertheless, amplified products are seldom detected by direct visualization in ethidium bromide-stained agarose gels, but rather by Southern blotting or dot blot hybridization (7, 30, 41). Although membrane hybridization is useful in research because it provides excellent sensitivity, these methods are generally time-consuming and labor intensive. These detection methods are therefore considered impractical for routine use in high-throughput laboratories.

Due to the ability of PCR to amplify specifically a gene or segment of a gene directly from a sample, an important factor in evaluating any DNA-based test is the specificity of the DNA sequence chosen for the genes and strains of interest. The primers selected for this study were based on the sequences of the *H. pylori glmM* gene. Investigation of 60 *H. pylori* isolates by PCR analysis and DNA sequencing indicated that the gene was present in every isolate (27). Furthermore, the *glmM* gene has recently been shown to be essential for *H. pylori* cell growth (15). PCR assays based on the *glmM* (*ureC*) gene have been described elsewhere (3, 7, 8) and were applied to the identification of *H. pylori* from bacterial colonies, gastric biopsies, feces, dental plaque, and oral rinses.

In this report, we have developed and evaluated a novel heminested PCR assay for the detection of *H. pylori* in clinical samples obtained from the oral cavity. In contrast to conventional nested and heminested PCR procedures (1) which are performed using two rounds of successive amplification consisting of 30 cycles each with an outer and an inner primer pair, respectively, this heminested PCR, based on amplification of the *glmM* sequence, is performed in a total of 30 cycles. Increasing the number of cycles often leads to the formation of nonspecific amplification products that result from mispriming within nontarget sequences encountered in the sample or by primer-dimer formation (26, 28, 42). The heminested PCR assay increased not only the specificity of the assay but also the detection limit. Only *H. pylori* strains screened by the PCR assay resulted in visualization of the predicted 496-bp amplified product in ethidium bromide-stained gels. Under the tested parameters, as little as 10 pg of purified chromosomal DNA, equivalent to 5×10^4 bacteria, was detectable by a single, first-round PCR assay. However, heminested PCR amplification enhanced the limit of detection considerably (at least 100-fold), and 0.1 pg of *H. pylori* DNA (5×10^2 organisms) was detectable in this assay. This is an improvement over an earlier study in which 100 pg of purified chromosomal DNA was needed for detection of *H. pylori* by PCR on the *glmM* gene (7).

The applicability of the heminested PCR procedure for screening oral specimens for the occurrence of *H. pylori* was also evaluated. The same series of saliva and dental plaque specimens were tested by heminested PCR and by a second PCR assay using oligonucleotide primers HPU50 and HPU25 because of their reported specificity in amplifying an *H. pylori*-specific 933-bp region of the urease B gene (13). The results indicated that one of three specimens found to be positive after amplification with the urease B gene-specific primers tested negative for *H. pylori* by the heminested PCR approach. Sequencing analyses of the amplicon indicated that it originated from *S. epidermidis*, a bacterium frequently associated with the oral environment.

These results not only confirm the specificity of the heminested PCR but they also highlight the importance of validating the identity of the amplification product by a second specific and sensitive method. This may be particularly important when clinical specimens are directly screened for the presence of *H. pylori*. Dental plaque may harbor more than 350 different bacterial species, and *H. pylori*-like organisms in the microflora may give false-positive PCR results on analysis of plaque material in some patients (36). In this study, the use of a heminested PCR minimized the chance of generating false-positive results, since any nonspecific amplicons produced during the first PCR step should not be able to function as target DNA during the second PCR step due to a lack of complementarity with the inner primer sequence, thereby making confirmation of the product by other procedures such as nucleic acid hybridization or sequencing unnecessary.

The possibility that *H. pylori* may colonize the oral cavity has attracted considerable attention. The presence of *H. pylori* in dental plaque of patients both with and without stomach disorders has been investigated by bacterial culture and PCR methods. Most studies have failed to isolate *H. pylori* by culture from dental plaque of subjects with gastric infection (4, 5, 6). PCR analysis of dental plaque from subjects with gastric infection has yielded more conflicting data, with *H. pylori* DNA found frequently in some studies (4, 35) but absent or found at extremely low frequencies in other studies (5, 24). In the dental plaque of healthy subjects, culture methods have detected *H. pylori* at a high frequency in a single study (32), while PCR has only very rarely detected *H. pylori* at high frequency (4). *H. pylori* has been detected by PCR in the saliva of symptomatic subjects (23, 31, 39), whereas culture methods have very rarely isolated *H. pylori* from saliva (19).

The low detection rate obtained in this study (2 of 58 samples) is in agreement with previous reports in which *H. pylori* was detected in 2 of 124 samples (8) and 0 of 10 samples (7). The low detection rate in this study may have been due to the culturing of oral samples prior to PCR analysis. Culture of *H. pylori* from patients' oral samples has been problematic because currently available culture media for isolation of *H. pylori* are frequently overgrown by other, faster-growing bacteria, and the prevalence of *H. pylori* in the oral cavity may be underestimated due to the presence of viable but nonculturable coccoid *H. pylori* organisms (9). In this regard, PCR assays have a much greater sensitivity, and we believe a greater degree of positivity may have been obtained if DNA were directly isolated from the specimens and then used in the heminested PCR procedure.

In conclusion, we consider the heminested PCR assay to be highly accurate for detection and confirmation of *H. pylori* infection based on the following reasons. First, PCR products that were positive by electrophoresis were also sequenced, which revealed those sequences were derived from *H. pylori* cells and not from other helicobacters or bacteria. Secondly, the PCR results could be reproduced by using the facilities of a different laboratory, thus validating the repeatability of the heminested PCR assay. The method could therefore have a potential value for further epidemiological studies for *H. pylori*-related research.

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