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Evaluation of Nested PCR in detection of *Helicobacter pylori* targeting a highly conserved gene: HSP60

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Abstract

Objective—To comparatively evaluate a new nested set of primers designed for the detection of *H.pylori* targeting a highly conserved heat shock protein gene (Hsp60).

Methods—A total of 60 subjects having peptic ulcer diseases were tested for the detection of *H.pylori* using rapid urease test (RUT), histology, culture and PCR in their antral biopsy specimens. A newly designed Hsp60 gene based primers set was evaluated against commonly used PCR primers for detection of *H.pylori*.

Results—Forty six of the 60 study subjects were found positive for culture isolation and all the 46 culture positive specimens were also positive with Hsp60 gene PCR. Of the 46 culture positive specimens, 44 were positive for 16S r RNA gene, *ureC* gene, RUT and histology while only 29 were positive with *ureA* gene PCR. Of the 14 culture negative subject, 10 were positive with 16S r RNA gene, 4 were positive with *ureC* (glmM) gene PCR and 2 were positive with RUT and 1 was positive on histology.

Conclusion—This study shows that nested amplification targeting Hsp60 gene is the most sensitive and specific with LR⁺ and LR[−] values of ∞ and 0 respectively when compared with the other 3 PCR methods. Also HSP60 gene specific nested protocol was the most appropriate for detection of *H. pylori* in clinical specimens. This is particularly valuable because it can be used as a non invasive method for detecting *H. pylori* infection in young children and also, in follow-up studies with peptic ulcer patients, on samples like feces and saliva.

Keywords

Helicobacter pylori; heat shock protein; Urease A gene

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Introduction

Helicobacter pylori is one of the most prevalent infectious agents across the globe (1). It has been implicated in the pathogenesis of active chronic gastritis, peptic and duodenal ulcer disease and gastric carcinoma (2,3). Because of its clinical importance, a large number of laboratories are detecting *H. pylori* routinely. Although, culture isolation has been the standard method for detection of infectious agents, yet it may not be the most appropriate method for bacteria like *H. pylori* which are both difficult and time-consuming to grow. Among the other methods, detection of a specific antibody against *H. pylori* may be irrelevant in a country like India where prevalence of this bacterium occur at endemic proportions (4). Rapid urease test (RUT) and histology may not prove to be specific tests since presence of other urease producing bacteria with *H. pylori* like morphology in stomach can not be denied (5). PCR based tests, which are able to detect very small amounts of nucleic acid, have been proved to be quite satisfactory for detection of such difficult organisms in clinical specimens. Target genes such as 16S r RNA (6), 26kDa species-specific antigen (SSA) (7,8), urease A (*ureA*) (9), urease C (*ureC*) (10), and random chromosome sequence (11) have been used for PCR based detection but with highly variable sensitivity and specificity. This variability may be attributed to inconsistency at the level of strains and also at the geographical level in the nucleotide sequences of *H. pylori* genome. Highly conserved sequences like 16S r RNA and urease may lack sufficient variability at the genus level. Similarly, two more target genes, the 26-kDa species-specific antigen SSA and glmM (phosphoglucomutase) are present in all the strains, but lack sufficient variability to develop a robust test. The putative virulence genes of *H. pylori* may not present in all the strains, so they are not suitable targets. Heat shock protein (HSP60) has been chosen as a target to develop a nested protocol to detect *H. pylori* in clinical specimens because it combines a high degree of conservation, presence in all biota (12) and sufficient variability to have species-specific primers. Already reported *H. pylori* specific primers targeting 16S r RNA, *ureA*, and *ureC* genes are available for comparison.

Material and Methods

The subjects were patients attending indoor services of the Department of Gastroenterology, University Hospital of Banaras Hindu University, Varanasi, Uttar Pradesh, India. This hospital provides tertiary level health services to the Eastern part of Northern India. Individuals who were already on antibiotic therapy or proton pump inhibitor (PPI) for previous 4 weeks were excluded from this study. The culture isolation and PCR related work was carried out in the Departments of Microbiology and Biochemistry, Institute of Medical Sciences, while primer designing and evaluation were carried out at Cell and Molecular Biology Laboratory, Sidney Kimmel Cancer Center, San Diego, CA.

Collection of Specimens

A total of 60 subjects having peptic ulcer (PUD) i.e. stomach mucosa discontinuity > 5 mm were included in the present study. Three biopsy specimens of antral tissue were collected by endoscopic forceps taking full aseptic precautions, from each individual after taking well-informed consent.

Work was approved by ethical committee, IMS, BHU.

Processing of specimens

The three biopsy specimens were pooled and ground together in an all glass disposable homogenizer and was divided into 3 aliquots. An aliquot of this tissue homogenate was transferred immediately into RUT medium and second part was plated within 30 minutes of collection, on media containing brain heart infusion agar (Difco, USA), supplemented with 7%

sheep blood, 0.4% IsoVitaleX and Skirrow selective supplement (vancomycin, 10µg/ml; polymixin B sulfate 2.5 IU/ml; trimethoprim lactate 5µg/ml) (Difco, USA). Plates were incubated at 37°C in an atmosphere of 5% O₂, 10% CO₂, and 85% N₂ for 3 to 7 days. *H. pylori* colonies were identified on the basis of their typical colony morphology, gram negative spiral rods and oxidase and urease production.

RUT

For RUT, biopsy specimens were inoculated into 1ml of 10% urea in deionized water (pH 6.8), to which two drops of 1% phenol red solution was added, and incubated at 37°C for 24 h. A positive result was recorded when the color changed from yellow to pink within 30 minutes. However if there was mild color change within 30 minutes, the RUT tubes were incubated for further 24 hours.

Histological Examination

Biopsy specimens were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. One 4-µm-thick section was cut and stained with hematoxylin-eosin to observe the presence of curved rod shaped bacteria on the mucosal surface.

Preparation of genomic DNA for PCR assay

Extraction of genomic DNA from both bacterial isolates as well as from tissue homogenate was done using a standard proteinase K and phenol-chloroform method (6)

Primers designed for HSP60 gene

Forward and reverse oligonucleotides were derived from the conserved region located between bases 8085 and 8675 of the HSP60 gene of *H. pylori* (ATCC 26695). An internal primer was derived from the region between bases 8162 and 8663 (Gene Bank: Accession no. NC-0091 ; gene ID 899089). For designing the *H. pylori* specific HSP60 gene primers, the HSP60 gene of *Helicobacter* (*H. pylori* 26695, *H. pylori*-J99) and related species (*H. hepaticus* ATCC 51449, *Wolinella succinogenes* and *Campylobacter jejuni* NCTC 11169) along with several unrelated bacteria were selected.

Bacterial strains

The two fully sequenced *H. pylori* reference strain DNA (J99 & ATCC 26695) were used as a positive control. The specificities of the four PCR methods were first examined for 6 different bacteria: *Campylobacter jejuni*, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC13883, *Proteus mirabilis* ATCC 7002, and biochemically and serologically confirmed clinical isolates of *Salmonella* Typhi and *Salmonella* Paratyphi A.

PCR amplification

PCR was carried out in a 25µl volume using 10ng of DNA, 1U of Taq polymerase (Bangalore Genie, India), and 10pmoles of each primer (Quiagen Operon, Cologne, Germany), 0.25mM (each) deoxynucleotide triphosphate (MBI, Fermentas) and 2-3mM MgCl₂ in standard PCR buffer. All the amplifications were carried out in a thermal cycler (Biometra, Goettingen, Germany). Details of primers and their protocol are shown in Table1. Amplification of the HSP60 specific sequence was done by nested protocol while for 16S r RNA, it was hemi-nested. Universal eubacterial primers were used for all the samples to exclude PCR inhibition. On the other hand single step PCR was carried out for *ureA* and *ureC* (*glmM*).

Analysis of the PCR amplified products

To identify the amplified products, 10 μ l of the PCR product was analyzed by electrophoresis on 1.5% agarose gels. (Bangalore Genie, India.)

Statistical Methods

For statistical analysis, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio for a positive test result (LR⁺), and likelihood ratio for a negative test result (LR⁻) were calculated (13)

Results

Of the 60 antral biopsy specimens collected from patients, 76 % (46/60) were found to be positive by RUT, 75 % (45/60) microscopy after hematoxylin-eosin and 76 % (46/60) by culture for *H. pylori* like bacteria. When these isolates were subjected to amplification by primers specific for 4 different genes of *H. pylori* i.e. 16S r RNA, *ure C*, HSP60 and *ure A* genes, all 46 were positive for the desired size of amplicon for the corresponding genes with the first 3 gene sequences. However, only 44 (95.6%) strains were positive for *ure A* gene specific amplification. Based on the positions of primers HSP1 and HSP2 within the nucleotide sequence of HSP60, a 590bp amplicon was generated from both the *H. pylori* reference stains (J 99 and ATCC 26695). Further, the primer pairs for the nested protocol designed in the present study could detect as little as 30 ng of template DNA on primary amplification and 1 fg (corresponds to approximately 3 organism) after the nested cycle, while the hemi-nested protocol targeting 16S r RNA could detect 0.01 pg. The two other PCR protocols targeting *ureA* and *ureC* used as single step amplification could detect 0.1 pg. However, amplification was negative with all the non *H. pylori* bacteria used in the present study i.e. *C. jejuni*, *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *P. mirabilis*, *S. Typhi* and *S. Paratyphi*.

When all the biopsy specimens i.e. 46 *H. pylori* culture positive and 14 negative for the isolation were subjected to amplification by universal eubacterial primers targeting 16S r RNA sequences, it was observed that 100% (60/60) were positive by universal eubacterial primers. Further, all the 46 specimens positive for culture isolation were also positive for desired amplicon by HSP60 specific primers (100%). However, 44 (95%) of the specimens were positive for the desired amplicon by both the genes of *H. pylori* i.e., 16S r RNA and *ure C* specific primers. The positivity observed with *ure A* gene specific primers was only 29 (63%)

Of the 14 culture negative specimens, 2 were positive by RUT and 1 by microscopy. Positivity for the specific amplicon using *H. pylori* gene specific primers was found in 10 cases with 16S r RNA and four with *ure C*. However, none of these 14 specimens was positive for HSP60 and *ure A* gene specific amplification.

Amplification by HSP60 and *ure A* was found to be the most specific (100%) followed by histology, RUT, *ureC* and 16S r RNA gene specific amplification at 92%, 85%, 71% and 28% respectively. The most sensitive method observed was HSP60 (100%) followed by histology, RUT, *ure C* and 16S r RNA based amplification, each at 95% followed by 63% for *ure A*. The PPV, NPV, LR⁺ and LR⁻ of each test are shown in table 3. LR⁺ was found to be ∞ for HSP60 and *ureA* gene specific amplification followed by 11.87, 6.3, 1.31 and 3.2 for histopathology, RUT, 16S r RNA and *ure C* based tests respectively. However, LR⁻ was found to be minimum for HSP60 with a value of 0.00 and 0.05, 0.05, 0.07, 0.1 and 0.37 for RUT, histopathology, *ure C*, 16S r RNA and *ure A* respectively in ascending order.

Discussion

H. pylori is known for its panmictic population. Different genes of *H. pylori* have been targeted for PCR based detection in biopsy tissue, saliva, dental plaque, and stool and in culture isolates, with variable specificity and sensitivity reported. In this study, we decided to target HSP60, which is known to be highly conserved but with sufficient variability to design species specific primers (10). The HSP60 specific primers screened in the present study seem to be very specific since none of the closely or distantly related bacterial DNA targets yielded positivity for the desired amplicon. The nested protocol showed the greatest sensitivity, detecting as little as 0.001pg of the *H. pylori* DNA. The lower sensitivities found by targeting *ureA* and *ureC* genes are to be expected as single step PCR was used. However, the hemi-nested protocol of 16S r RNA also had lower sensitivity than that for HSP60.

For a test to be accepted as clinically useful, it is imperative that the LR⁺ and LR⁻ of the test should be >10 and < 0.1 respectively when analyzed relative to a gold standard (11). In the present study, HSP60 based amplification was the best with LR⁺ and LR⁻ values of ∞ and 0 respectively, when culture isolation was used as the gold standard. Although none of the other three PCR based techniques could reach this standard, histopathological examination was found to be quite satisfactory, with LR⁺ and LR⁻ values of 11.87 and 0.05 respectively. The poor specificity 16S r RNA gene PCR may be explained by sequence conservation across the bacterial genera and also by possible amplification of nonspecifically human DNA (14,15). The reasons for poor sensitivity of detection of *H. pylori* DNA by *ureA* and *ureC* (glmM) may be that both of them are single step PCR and thus unable to detect the lower number of *H. pylori* and also that they were unable to counteract PCR inhibitors in the clinical specimens. Relatively lower NPV observed in both of these tests suggest that the sequences are very much conserved but the conservation might be beyond the species and even the genera (16)

However no amplification by *ure A* gene specific primer in 2 of the 46 strains suggests the possibility that this gene either may not be present in all the strains or that it may have sufficient polymorphism in the loci for proper annealing with the primers.

In conclusion, of the methods studied, HSP60 gene specific nested protocol was the most appropriate for detection of *H. pylori* in clinical specimens. This is particularly valuable as it can be used as a non invasive method for detecting *H. pylori* infection in young children and also, in follow-up studies with peptic ulcer patients, on samples like feces and saliva. However, non specific tests like RUT and histopathology can also be relied upon for detection of the bacterium in antral biopsy where PCR based methods are not feasible.

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Approval for this was given by our local ethical committee:

Dean, Institute of Medical Sciences, Banaras Hindu University, Varanasi,(UP.) India.

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Table 1

Conditions for the four different PCR methods

Target gene and size of products	Primer names and sequences	PCR condition & (annealing temp.& cycles) MgCl ₂ conc.
16S rRNA (6) 446 bp	Hp1, 5'-CTGGAGAGACTAAGCCCTCC-3' Hp3, 5'-AGGATCAAGGTTTAAGGATT-3'	95°C, 30s; 55°C, 30s; 72°C, 30s (30 cycles) (2.5mM MgCl ₂)
110 bp	Hp1, 5'-CTGGAGAGACTAAGCCCTCC-3' Hp2, 5'-ATTACTGACGCTGATTGTGC-3'	95°C, 30s; 62°C, 30s; 72°C, 30s (30 cycles) (2.5mM MgCl ₂)
UreA gene (9), 411 bp	HPU1, 5'-GCCAATGGTAAATTAGTT-3' HPU2, 5'-CTCCTAATTGTTTTTAC-3'	94°C, 1 min; 45°C, 1 min; 72°C, 1 min (35 cycles) (2.5mM MgCl ₂)
<i>glmM</i> gene (10), 294 bp	5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' 5'-AAGCTTACTTTCTAACACTAACGC-3'	93°C, 1 min; 55°C, 1 min; 72°C 1 min (35 cycles) (2.5mM MgCl ₂)
HSP60 primer (designed for this study) 501bp	HSP ₁ 5'-AAGGCATGCAATTTGATAGAGGCT-3' HSP ₂ 5'-CTTTTTTCTCTTTCATTTCCACTT-3' HSPN ₁ 5'-TTGATAGAGGCTACCTCTCC-3' HSPN ₂ 5'-TGTCATAATCGCTTGTCGTGC-3'	94°C, 30 s, 56°C, 30 s ;72°C 30s (30cycles) (2.5mM MgCl ₂) same cycle condition.
Universal eubacterial (6) 1,156bp.	5'-CGGTTACCTTGTTACGACTT-3' 5'-CAGTGGGGAATATTGCACAA-3'	95°C, 30s; 55°C, 30s; 72°C, 30s (35 cycles) (2.5mM MgCl ₂)

Table 2
Result of four PCR methods for the detection of *H. pylori* in 60 gastric biopsy specimens.

No. of specimens	Culture	HSP60 gene	16S r RNA gene	<i>ure C (glmM)</i> gene	<i>ureA</i> gene	RUT	Histology
29	+	+	+	+	+	+	+
10	+	+	+	+	-	+	+
5	+	+	+	+	-	+	+
2	+	+	-	-	-	-	-
7	-	-	+	-	-	-	-
2	-	-	+	-	-	+	-
1	-	-	+	-	-	-	+
4	-	-	-	+	-	-	-

Table 3

Comparative evaluation of different tests considering culture isolation as gold standard.

	Hsp60	16Sr RNA	ureC	ureA	RUT	Histology
Sensitivity	100	95	95	63	95	95
Specificity	100	28	71	100	85	92
PPV ^a	100	81	95	100	95	97
NPV ^b	100	66	83	45	85	86
LR+	∞	1.31	3.2	∞	6.3	11.87
LR-	0	0.1	0.07	0.37	0.05	0.05

^a Compared with 46 culture-positive samples.^b Compared with 14 culture-negative samples.