Original Article

Detection of *Helicobacter pylori* in stool specimens: comparative evaluation of nested PCR and antigen detection

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Abstract

Background: Efficacy of *Helicobacter pylori* stool antigen enzyme immunoassay (HpSA) and stool PCR was evaluated, before and after treatment, in a country with a high prevalence of *H. pylori* infection.

Methodology: A total of 52 patients with dyspeptic symptoms were included in the study. Antral biopsy was collected during pre- and post-therapy periods for rapid urease test (RUT) and PCR. Similarly stool specimens for PCR and HpSA test were collected during both the periods from all 52 patients. Biopsy, PCR and RUT results together were considered the "gold standard."

Results: On the basis of gold standard tests, 40/52 patients were *H. pylori* positive. The sensitivity and specificity of HpSA test were 80% and 83.3% respectively in untreated patients. On the other hand, the sensitivity and specificity of stool PCR in untreated patients were 72.5% and 100% respectively. After eradication therapy, the results of both RUT and biopsy PCR were negative in 87.5% and positive in 12.5% of the patients. Although post treatment sensitivity of HpSA and stool PCR was equal (60%), specificity of HpSA and stool PCR were 68.6% and 97.1% respectively.

Conclusion: The *H. pylori* stool tests represent a non-invasive concept for diagnosis of infection. Both HpSA and stool PCR seem to be satisfactory tests for pre-eradication as well as assessment of infection. But stool PCR is a better indicator than HpSA test in the post-eradication assessment of infection.

Key Words: *H. pylori*, HpSA, Stool PCR, Biopsy PCR, RUT, *hsp*.

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Introduction

Helicobacter pylori have been linked to gastritis, duodenal ulcer, gastric carcinoma and mucosa associated lymphoid malignancies [1, 2]. More than 50% of the world's population, and as many as 80% of developing-country residents carry *H. pylori*. Serological studies performed in the Indian subcontinent indicate a prevalence of 22% to 57% in children under 5 years of age, and increasing to 80% to 90 % by the age of 20 years and remaining constant thereafter [3,4,5]. However, serological tests are reported to be unreliable for the diagnosis of *H. pylori* since they may return false negative results up to 60 days after infection and remain positive for a considerable time after eradication. The urea breath test, although non-invasive and reliable, is expensive and technically demanding. Isolation of bacteria from the antral region is ideal but it requires invasive procedures and has poor sensitivity due to the fastidious nature of *H. pylori*. There is evidence that *H. pylori* is excreted in the feces of infected individuals. The pathogen can be detected in stool specimens by PCR [6], and rarely by culture [7,8]. PCR based detection of *H. pylori* has proved to be a very powerful technique, but its efficacy is marred by fecal PCR inhibitors and the relatively lower number of bacteria in the colon. We have designed and evaluated a nested PCR protocol targeting the conserved heat shock protein (*hsp*60) gene, which can detect as little as 30 ng of template DNA from culture isolate following primary amplification and 1 femtogram after the nested PCR cycle. The *hsp*60 specific primers are specific since no closely, or distantly, related bacterial DNA targets produced the desired amplicon. [9]. An enzyme immune assay based detection of *H. pylori* has provided an alternative non-invasive method to detect the bacterium in stool specimens. In the present study, therefore,

we planned to evaluate the diagnostic utility of both of the PCR and antigen based detection system in stool tests before and after treatment.

Materials & Methods

The subjects included in this study were the 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. The PCR and antigen detection related work was conducted in the Departments of Microbiology and Biochemistry, Institute of Medical Sciences.

Collection of specimens

Fifty-two consecutive outpatients participated in this study. There were 35 males (67.3%) and 17 females (33.7%) enrolled. Approval for this study was given by the Ethical Committee of the University Hospital of Banaras Hindu University, Varanasi, Uttar Pradesh, India.

The ages of the patients ranged from 16 to 62 years (median age 44.6 years). Patients had been referred to endoscopy because of various gastrointestinal symptoms. Three biopsy specimens of antral tissue were collected from each individual after well-informed consent was given. Individuals who received any antibiotics or proton pump inhibitors during the previous one week and those who received H2 receptor antagonist or antacid treatment during the previous 24 hours were excluded from the study. Stool samples were taken twice during the study, i.e. once before the start of the eradication therapy and the other after 6 weeks of completion of therapy. A patient was considered as *H. pylori* infected if both rapid urease test (RUT) and biopsy PCR were positive, and as *H. pylori* negative when both tests gave negative results. Patients with positive results for *H. pylori* were treated with a combination of clarthiromycin (500mg), amoxycillin (1g) and omeprazole (20mg); all were given twice daily for 14 days.

DNA analysis

Extraction of genomic DNA from biopsy was performed sequentially with sodium dodecyl sulfate, proteinase K, and hexadecyltrimethyl ammonium bromide (CTAB). The crude extract was purified by phenol-chloroform extraction and PCR-inhibitors were removed as described by Van Zwet *et al*. [10] with slight modifications.

PCR amplification

PCR was performed in a thermocycler (Biometra, Germany) according to standard procedures. To ensure that bacterial DNA from each sample was available for PCR amplification of the *hsp*60 gene, each sample was subjected to PCR amplification using universal eubacterial primers: f 5'-AGGAGGTGATCCAACCGCA-3' and r 5'-AACTGGAGGAAGGTGGGGAT-3' [11]. Each reaction was carried out in a 50 µl reaction volume, containing 15 pmol of each primer, 0.25mM (each) dNTP (MBI, Fermentas) and 3.0mM MgCl2 in the proprietary buffers, 1U Taq DNA Polymerase (Bangalore Genie, India) and 2 µl of the extracted template. The reaction comprised 40 cycles of 30 seconds at 95° C followed by 30 seconds at 55° C with a final extension step of 10 minutes. The PCR product was 370 bp.

2) *H. pylori* specific primers: The reaction was performed in 25 µl final volume containing 10ng of DNA, 1U of Taq polymerase (Bangalore Genie, India), 0.25mM (each) deoxynucleotide triphosphate (MBI, Fermentas) and 2-3mM $MgCl_2$ in standard PCR buffer and 10 pmoles of each primer [9].

Primers

HSP1 (5'-AAGGCATGCAATTTGATAGAGGC T-3') and HSP2 (5'-CTTTTTTCTCTTTCATTTC CACTT-3), generated 590bp amplicon from *H. pylori* reference strain J99. The primers HSPN1 (5'-TTGATAGAGGCTACCTCTCC-3'), and HSPN2 (5'-TGTCATAATCGCTTGTCGTGC-3') were used to amplify a 501 bp internal fragment of the *hsp*60 gene of *H. pylori*. Initial denaturation was conducted for 5 minutes at 95º C and cycling was performed as follows: 94º C for 30 seconds, 56º C for 30 seconds, 72º C for 30 seconds and 72º C for 10 minutes for 30 cycles. For the internal amplification, the PCR product from the primary cycle was diluted 1/50 and 1µl was used as the template in the nested PCR. The conditions for the PCR amplification, first reaction and second cycles were the same. DNA from *H. pylori* reference J99 and a tube containing water in place of DNA were

assayed in each PCR run as positive and negative controls, respectively.

In the case of PCR for stool samples, absence of PCR inhibitors were verified by repeating PCR after adding a known amount (10^3) of *H. pylori*.

Detection and analysis of amplified PCR product

Southern hybridization

Ten microliter amounts of each PCR product were electrophoresed on agarose of 1.5% gel (Bangalore Genie, India) which was stained with ethidium bromide and visualized under UV light. Southern blots were performed on randomly selected PCR positive samples to confirm that the amplified product was from *H. pylori*. The amplified fragments were transferred from the agarose gel to a nylon membrane (Genie southern hybridization Kit, Bangalore, India) and hybridized according to the manufacturer's protocol. The PCR product of the secondary cycle of the nested PCR (with DNA of *H. pylori* reference strain J99 as the template) was labeled with biotin -11-dUTP by using a Biotin Decalabel DNA labeling Kit (Fermentas, USA). The hybridized probe was detected by incubating the membrane with streptavidine-HRP (Horse radish peroxidase) conjugate and finally incubated with the substrate TMB/H_2O_2 (Tetramethyl benzidine H_2O_2 substrate) that reacted with HRP to give a blue colour band on the nylon membrane.

Cloning and sequencing

The amplified product of expected size was purified from agarose gel using a PCR Purification Kit (Quiagen, Valencia, CA, USA). Purified PCR products with *hsp*60 primers were cloned into the pGEM-T Easy vector by using a PCR cloning kit (Promega, Madison, USA). Plasmid DNA was extracted using a Plasmid Purification Kit (Quiagen, Valencia, CA, USA) and outsourced for sequencing (Bangalore Genie, India). Sequences were analyzed using BLAST N (http://www.ncbi.nlm.nih.gov/BLAST/) to verify identity of the source organism as *H. pylori*.

Stool antigen detection

HpSA (Premier Platinum HpSA, Meridian Diagnostics, Cincinnati, OH, USA) was performed strictly following the instructions of the manufacturer without knowledge of the *H. pylori* status. In brief, after thawing at room temperature, a small quantity of stool sample, 5–6 mm in diameter, was mixed with 200 µl of sample diluent. Fifty microliters of the diluted fecal sample were added to a microwell to which a polyclonal antibody against *H. pylori* was absorbed. Anti *H. pylori* and peroxidase conjugated antibody was added. The microwells were kept undisturbed for one hour and then rinsed thoroughly to remove unbound material. Subsequently, a substrate-containing buffer solution was added to the microwell and the microplate was incubated for 10 minutes at room temperature. The reaction was stopped by adding 1M sulfuric acid (pH ≤ 1.0). The yellow color was spectrophotometrically read at a wavelength of 450 nm. An optical density OD_{450} < 0.140 indicated the absence of *H. pylori* infection. An optical density from 0.140 to 0.160 is equivocal. Value ≥0.160 indicated the presence of *H. pylori* antigens.

Statistical analysis

For statistical analysis sensitivity, specificity, 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 [12].

Results

Biopsy specimens subjected for evaluation of different diagnostic tests from 52 patients revealed that 41 (78.8%) were positive by RUT and 40 (76.9%) by PCR. With the exception of a single case positive by RUT, the other 40 cases could be detected by both of the tests. The test based on antigen detection of *H. pylori* in stool, HpSA, could detect *H. pylori* in 65.3% (34/52) of the samples collected before therapy, whereas the detection rate by PCR in stool specimens had been 55.8 % (29/52). Of the 5 HpSA positive cases which could not be detected by stool PCR, 3 were positive both by RUT and PCR in antral biopsy. However, 2 of them were exclusively positive by HpSA. None of the stool samples could be found equivocal by HpSA in the present study. The 12 patients that were negative for the biopsy PCR test were also negative by the stool PCR. However, 2 of these 12 patients were positive by HpSA. When follow-up 6 weeks after the triple therapy was completed, the results of both the RUT and biopsy PCR were negative in 87.5 % (35/40) of the patients, while the remaining 12.5 % (5/40) of the patients were positive by both, and 4 (7.6%) of the 52 stool samples collected after eradication therapy could be detected positive for H *pylori* specific gene PCR

amplification. These 4 cases were also positive by HpSA. However, 10 stool specimens were observed to be positive exclusively by HpSA collected after eradication therapy.

Southern blot analysis of the amplicon obtained from feces and antral biopsy specimens confirmed that these amplified products were from *H. pylori*. Also, cloning and DNA sequence analysis of these amplicon yielded sequences which could be aligned with the nucleotide sequence of *hsp*60 gene of *H. pylori* (ATCC26695) with a similarity of > 97%.

Table 1 shows that the sensitivity, specificity, PPV and NPV of HpSA based detection of *H. pylori* in the pretreatment period had been 80%, 83.3%, 94.1% and 55.5% respectively with the LR^+ and LR^- values of 4.8 and 0.24. Nested PCR based detection of H. *pylori* in stool demonstrated 72.2% sensitivity, 100% specificity, 100% PPV, 52.2% NPV, LR^+ ∞ and LR^- 0.27 respectively during the pre-treatment period. However, the sensitivity, specificity, PPV, NPV, LR^+ and LR^- values for HpSA in the post-treatment period were 60%, 68.6%, 21.4%, 92.3%, 1.91 and 0.58, respectively. On the other hand, the same parameters for stool PCR were 60%, 97.1%, 75% and 94.4% respectively with the LR^+ and LR^- values of 28.2 and 0.41.

Table 1. Comparative evaluation of HpSA and stool PCR during pre and post eradication therapy for *Helicobacter pylori*.

Pre-treatment	TP	FN	TN	FP	Sensitivity (%) (95%CI)	Specificity (%) (95%CI)	PPV (%)	NPV (%)	LR+	LR-
HpSA (n=52)	**32**	8	10	2	80 (65.5-90.2)	83.3 (54.9-97.1)	94.1	55.5	4.8	0.24
Stool PCR (n=52)	**29**	11	12	0	72.5 (57.2-84.6)	100 (77.9-100)	100	52.2	∞	0.27
Post-treatment	**TP**	**FN**	**TN**	**FP**	**Sensitivity (%) (95%CI)**	**Specificity (%) (95%CI)**	**PPV (%)**	**NPV (%)**	**LR+**	**LR-**
HpSA (n=40)	**3**	2	24	11	60 (18.2-92.6)	68.6 (51.9-82.2)	21.4	92.3	1.91	0.58
Stool PCR (n=40)	**3**	2	34	1	60 (18.2-92.6)	97.1 (86.7-99.8)	75	94.4	28.2	0.41

Note: PCR and RUT positivity in antral biopsy for *H. pylori* is taken as gold standard. TP: True positive, TN: True negative, FP: False positive, FN: False negative, PPV: Positive predictive value, NPV: Negative predictive value, LR+: Likelihood ratio for a positive test result, LR- : Likelihood ratio for a negative test result.

Discussion

In the current study, the sensitivity, specificity and positive and negative predictive values of stool PCR in untreated patients were 72.5%, 100%, 100% and 52.2% respectively. In this study, the sensitivity of PCR from stool specimens exceeds that of previously reported attempts [13]. Therefore, stool PCR may be considered as better test than HpSA because the HpSA test shows heterogeneity in the sensitivity rates that ranged from 58% to 96% and specificity ranged from 67% to96 % [14-22]. This is because of the difficulty in obtaining polyclonal antibodies of constant quality [23].

In the present study, stool PCR positivity (7.5%, 3/40) corresponded very closely to antral biopsy PCR (12.5%, 5/40) in post-treatment testing. Although the same 3 cases were also positive by enzyme immune assay based detection system in stool (HpSA) in the post-treatment testing, there were 11 false positive cases, also bringing the specificity of this HpSA to the level of 68.6% when antral biopsy PCR and RUT were taken as gold standard. The probable cause of false positive results in the case of the HpSA test could be the cross-reactivity with other species of *Helicobacter* that colonize humans [24]. Furthermore, in pre-treatment testing, the specificity of this test had been only 83.3% in comparison to 100% by stool PCR. The specificity of stool PCR in the post-treatment test again was found to be 97.1%, which is much better than that of HpSA (68.6%). However, the sensitivity of HpSA was found to be marginally better than the stool PCR during pre-treatment, i.e. 80% and 72.5%, but the same (60%) during post treatment tests. To assign a test to be of clinical utility, it is recommended that the LR^+ and LR^- of the test should be 10 and 0.1, respectively [12]. In pre-treatment testing the LR^+ and LR^- values for HpSA had been 4.8 and 0.24 while for the stool PCR the LR^+ and LR^- values had been ∞ and 0.27 respectively. Similarly, post-therapy LR^+ and LR^- for HpSA had been 1.91 and 0.58 while for stool PCR the LR^+ and LR^- had been 28.2 and 0.41. These results also suggest that stool PCR seems to be a better test. Stool tests can be used as a non-invasive tool to assess *H. pylori* status. Stool samples are easy to collect, even for unskilled personnel or the patients themselves. In conclusion, HpSA and stool PCR are valuable

tests in the pre-eradication assessment of infection. Also, they could be used in epidemiological studies to determine the prevalence of *H. pylori* infection in symptomatic and asymptomatic subjects. Stool PCR is a more valuable test than HpSA in the post-eradication assessment of infection.

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