

# Evaluation of Molecular Technique over Conventional Culture Method for Early Diagnosis of *Helicobacter pylori* Infection

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#### ABSTRACT

**Background:** *Helicobacter pylori* infection is an important triggering agent for development of gastric malignancies, peptic ulcer disease and other dyspeptic symptoms. Therefore a crucial part in the management of infection is a fast and accurate diagnostic technique which would give reliable results in detecting infection in symptomatic patients.

**Aim:** This study aimed to compare and evaluate the reliability of molecular technique over conventional culture method for early diagnosis of *H. pylori* infection in dyspeptic patients.

**Materials and Methods:** A cross sectional hospital-based study was conducted between June 2016 and May 2017 and included 100 gastric biopsy specimens from clinically suspected dyspeptic patients undergoing routine upper gastrointestinal tract endoscopy. These specimens were analyzed using the conventional culture method as well as molecular technique (PCR assay) for species identification and virulent factor determination directly from biopsy specimens.

**Results:** The molecular method could detect *H. pylori* in 44% cases with detection virulent *cagA* and *vacA* genes, whereas the culture method could detect 20% cases. Moreover the turnaround time taken by molecular method was 24-36

#### INTRODUCTION

Helicobacter pylori are a spiral, micro-aerophilic, Gram negative bacteria which colonises the human gastric mucosa in almost half of the world's population.<sup>1</sup> This bacterium is responsible for a number of stomach disorders such as chronic gastritis and peptic ulcer disease and might also be a triggering agent for various kinds of gastric lymphomas.<sup>2</sup> In the year 1994, the World Health Organization (WHO) declared H. pylori a human carcinogen.1 Patients infected with H. pylori develop chronic gastric inflammation which might remain initially asymptomatic. These conditions may later on eventually progress and increase the risk of peptic ulcer disease as well as gastric carcinoma.<sup>1</sup> Therefore a crucial part in the management and diagnosis of such gastrointestinal diseases is a fast, effective and accurate diagnostic method which would serve as a gold standard in the management of infection. Various kinds of treatments are available nowadays against H. pylori and early detection treatment Hours whereas that of the culture method was 5-6 days. **Conclusion:** The molecular method can be considered as a more sensitive as well as rapid method for detection of *H. pylori* from biopsy specimens which can be used routinely for diagnosis of *H. pylori* infection.

**Keywords:** Gastro-duodenal diseases, diagnostic accuracy, PCR, turnaround time, virulent genes

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will reduce the risk of developing gastric ulceration as well as gastric carcinoma. For detection of any micro-organism, culture method is considered as the gold standard technique, since culture isolates can be characterized in detail.<sup>3</sup> But culturing of organisms like *Helicobacter pylori* is often difficult as false negative results may arise due to its patchy distribution in the mucosa of the human stomach, its fastidious, micro-aerophilic nature and requirement of lengthy culture times.<sup>4,5</sup> For detection of this type of organism, a test is required which is more sensitive and specific and takes less time for early detection of *H. pylori* directly from gastric biopsy specimens. Infection with *H. pylori* can be detected directly by PCR from gastric biopsy specimens.<sup>6</sup>

This method detects one of the most important housekeeping genes of *H. pylori*, the *ureA* gene, responsible for urease production as well as genes associated with *H. pylori* virulence, mainly the cytotoxin - associated antigen (*cagA*) gene

and vacoulating cytotoxin gene (vacA). The cagA gene, present in about 60% of *H. pylori* strains induces morphological alterations as well as interleukin-8 secretion in the gastric epithelial cells. The vacA gene, which is present in almost all H. pylori strains, encodes a protein toxin which induces the formation of vacuoles in primary gastric epithelial cells, thus causing injury to the gastric epithelium.<sup>6</sup> The vacA gene contains at least two variable regions, the signal region(s) and middle region (m) and three allelic s subtypes called s1a, s1b, and s2, and two m types, called m1 and m2.6 The presence of the cagA and vacA genes in H. pylori infected patients is associated with more severe forms of gastro duodenal disease than any other clinical outcome.5, 6 Genotypic characterization of these genes can be performed directly from gastric biopsy samples without the need for culture which would save a considerable amount of time for effective diagnosis of H. pylori.5, 7, 8 Several studies have shown the molecular method to be more sensitive and efficient than the conventional culture method for *H. pylori* diagnosis.<sup>5,8-10</sup>

Keeping in view the substantial rise in *H. pylori* infection in spite of various available diagnostic methods, we aimed to evaluate and compare the efficacy of the PCR assay with conventional culture method for *H. pylori* diagnosis from gastric biopsy specimens from dyspeptic patients of Assam, North-east India. Although infection with *H. pylori* can be detected by a variety of methods, our present study aimed to compare the accuracy of two different diagnostic methods for diagnosis of *H. pylori* infection. These tests included the molecular method (PCR assay) and the conventional culture method.

### MATERIALS AND METHODS

Patients and Study Design: A cross sectional hospital based study was conducted between June 2016 and May 2017, where 100 dyspeptic patients of all age groups were subjected to routine upper gastro-intestinal endoscopy for suspected gastritis, peptic ulcer disease and gastric carcinoma out of which 73 were male patients with a mean age of 43.11 ± 16.49 years and 27 were females with a mean age of 37.96 ± 13.1 years. Out of 100 patients, 18 were suffering from peptic ulcer disease, 32 from chronic gastritis and duodenitis, 38 had non-ulcer dyspepsia and 3 had gastric cancer. Remaining 9 patients were grouped into 'Others' which included GERD, stomach and oesophageal varices, oesophageal growth. An informed written consent was obtained from each patient included in the study prior to sample collection. The study was approved by the Institutional Ethics Committee (Human) of our institution prior to initiation of the work and was carried out according to the guidelines of the Committee. Sample Collection: Two gastric biopsy specimens from antrum area of the stomach were obtained from each patient, placed into a transport media containing 0.6 ml Brain Heart infusion (BHI) broth (BD Difco Laboratories) and glycerol and were transported to the microbiology laboratory. Out of the two samples, one was transferred to a tube containing 80 µl phosphate-buffered saline (PBS) and processed for extraction of genomic DNA. The other sample was processed for culture in H. pylori specific media. Sample processing was done within two hours of collection. Sample Processing and Culturing Conditions for H. pylori Detection: For culturing of H. pylori, the patients' samples were processed and plated in H. pylori specific media within 2 hours on the day of sample collection. The biopsy samples contained in the transport media were vortexed at full speed to release the bacteria

from the mucosal surface. About 200 µl of the processed tissue sample was spread with a micropipette onto petriplates containing brain heart infusion (BHI) agar (BD-Difco Laboratories) with 7% sheep blood, Isovitalex enrichment (BD-Difco) and Dent supplement (Oxoid, England). The plates were incubated at 37°C for 5 to 6 days under microaerophilic conditions (Anoxomat MART Microbiology) containing 5-6% O<sub>2</sub>, 10-12% CO<sub>2</sub>, and 85% N<sub>2</sub> with 95% humidity. Following growth of H. pylori colonies on the culture media, they were subcultured on BHI agar. These were then further confirmed by biochemical tests such as urease, oxidase and catalase test. The incubation of the samples for appearance of visible H. pylori colonies followed by confirmation of results by biochemical tests was done approximately on the 5th day. Preparation and delivery of reports was done on the 6th day. The samples were considered culture positive for H. pylori, if smooth, grey, water droplet-like colonies, morphologically Gram-negative, comma or 's' shaped and were urease, oxidase and catalase test positive were visible on the culture media on the 5th or 6th day of incubation.

**Genomic DNA Extraction from the Gastric Biopsy Samples:** Following collection of the patients' biopsy samples on day 1, extraction of genomic DNA from the samples was done within 2 hours of sample transportation to the microbiology laboratory. Genomic DNA from the gastric biopsy samples was extracted by the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the instructions given by the manufacturer. The extracted DNA was processed for PCR.

**Polymerase Chain Reaction (PCR):** On day 2, PCR of *H. pylori ureA* gene as well as virulence factors *cagA*, *vacA* s1/s2, *vacA* m1/m2 regions of *H. pylori* was done directly from the gastric biopsy samples of the patients. These genes were amplified using published sequences of *H. pylori* specific oligonucleotide primers (Table 1).

PCR assay of all the genes was carried out in a final volume of 20  $\mu$ l containing 10-50 ng of genomic DNA, 10  $\mu$ M of primers, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M (each) deoxynucleotides, 2U Tag polymerase enzyme (all from Sigma Chemicals Co., St. Louis, MO) in a standard PCR buffer.

**PCR conditions of UreA gene:** A singleplex PCR assay was carried out to amplify a sequence of 411 bp (Figure 1) of the *ureA* gene of *H. pylori* using the following cycling conditions: Initial denaturation at 96°C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 45 °C for 30 sec, extension at 72 °C for 30 sec, and final extension at 72 °C for 4 min. PCR conditions of *CagA*, *VacA* s1/s2, *VacA* m1/m2.

For detecting *H. pylori* pathogenesis in the patients' samples, a sequence of 350 bp of the *cagA* gene, 259/286 bp of the *vacA* signal sequence (s1/s2) and 567/642 bp of the *vacA* mid-region (m1/m2) alleles of *H. pylori* was amplified by multiplex PCR assay (Figure 2) in a single reaction using the following cycling conditions: Initial denaturation at 96°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55 °C for 45 sec, extension at 72 °C for 1 min, and final extension at 72°C for 5 min. This assay was done immediately after species identification by *H. pylori ureA* PCR. In the PCR assays, genomic DNA from *H. pylori* 26695 and nuclease free water was used as positive and negative control respectively. The PCR products were analyzed by 2% agarose gel stained with ethidium bromide and observed under UV gel documentation system using 100 bp molecular markers (G-Bioscience, MO, U.S.A.).

Region amplified	Primer sequence (5´- 3)	Product size (bp)	References	
UreA	GCCAATGGTAAATTAGTT	411	22	
	CTCCTTAATTGTTTTTAC			
CagA	GTTGATAACGCTGTCGCTTC	350	23	
	GGGTTGTATGATATTTTCCATAA			
VacA s1/	ATGGAAATACAACAAACACAC	259/286	23	
VacA s2	CTGCTTGAATGCGCCAAAC			
VacA m1/	CAATCTGTCCAATCAAGCGAG	567/642	23	
VacA m2	GCGTCAAAATAATTCCAAGG			

#### Table 1: Oligonucleotide Primers used for PCR amplification

S.	H. pylori detection rate	Clinical condition			Total		
No		NUD <sup>a</sup> (%)	CG/D <sup>b</sup> (%)	PUDº (%)	GC <sup>d</sup> (%)	Others (%)	(n=100)
1	PCR positive, culture negative	15 (34.09)	17 (38.63)	9 (20.45)	1 (2.27)	2 (4.54)	44
2	Both culture & PCR positive	3 (15)	7 (35)	9 (45)	1 (6.66)	0	20
3	Both culture & PCR negative	20 (55.55)	8 (22.22)	0 (0)	1 (2.77)	7 (19.44)	36

# Table 2: U mylari dataction in different clinical conditions

a Non-ulcer dyspepsia; b Chronic gastritis/duodenitis; c Peptic ulcer disease (gastric and duodenal ulcer); d Gastric cancer "Others" include: GERD, stomach and oesophageal varices, oesophageal growth.

Virulent Genes	CagA	VacA- s1m1	VacA- s1m2	Both Cag A & Vac A
Positive	34	27	6	27
%	77.27	61.36	13.63	61.36

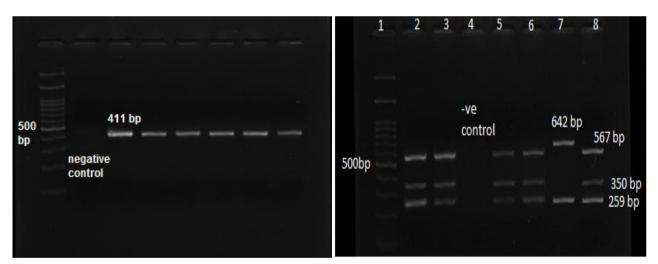


Figure 1: Amplification of *H. pylori UreA gene*. Lane 1- 100 bp DNA ladder. Lane 2- negative control. Lane 3 to 8 contains *UreA* genotype at 411 bp.

Figure 2: Amplification of VacA m1, VacA m2 and VacA s1 alleles and the CagA gene of *H. pylori* by multiplex PCR assay. Lane 1- 100 bp DNA ladder. Lane 2- positive control. Lanes 3, 5, 6 and 8- VacA s1m1 CagA genotype at 259 bp, 567bp and 350 bp respectively. Lane 7- VacA s1m2 genotype at 259 bp and 642 bp respectively. Lane 4- negative control.

# RESULTS

Out of 100 cases, both culture and PCR method could detect *H. pylori* in 20 numbers of cases, whereas the only PCR positive and culture negative method could detect *H. pylori* in 44 numbers of cases. None of the culture positive cases showed PCR negative results. Out of 20 culture and PCR positive patients, 9 had peptic ulcer disease, 7 had chronic gastritis/duodenitis, 1 patient had gastric cancer and 3 were non-ulcer dyspeptic patients (Table 2). Out of 44 PCR positive cases, 9 had peptic ulcer disease, 17 had

chronic gastritis/duodenitis, 15 had non-ulcer dyspepsia, one patient had gastric cancer and remaining 2 patients were grouped into "others". Thirty six (36%) cases were negative for *H. pylori* infection since they could not be detected by culture as well as PCR. Out of 36, 8 had chronic gastritis/duodenitis, 1 had gastric cancer, 20 were non-ulcer dyspeptic patients and 7 belonged to the "others" group. The detection of *H. pylori* in different clinical conditions in both culture and PCR is summarized in Table 2.

Table 3 shows the distribution of *H. pylori* virulent genes in PCR positive patients. Thirty four patients out of 44 (77.27%) patients were found to harbour the virulent cagA gene and 33 out of 44 patients (75%) harboured the vacA gene. VacA s1m1 allele was detected in 27 patients and s1m2 in 6 patients. VacA s2 allele was not detected in our study. Twenty seven (61.36%) patients were positive for both *cagA* and *vacA* genes. The average turnaround time for *H. pylori* detection via the conventional culture method, starting from the day of sample collection till the delivery of results was 6 days. Whereas, the molecular analysis of the patients' samples had a turnaround time of 24-36 hours. Thus keeping in view the lesser turnaround time required by the molecular method than the conventional culture method for delivering the results, as well as identification of a significant number of *H. pylori* infections that could not be detected by culture (false negative results), the efficacy and accuracy of the molecular method was shown to be much higher than the culture method.

## DISCUSSION AND CONCLUSION

In the present study, the molecular method identified a significant number of H. pylori infections that could not be detected by culture. It also detected putative virulence markers of H. pylori, the cagA and vacA genes indicating that this assay can be well applied for detection of *H. pylori* as well as virulent factors. Also this method showed high degree of efficacy than the culture method, owing to its faster turnaround time which yielded results within hours as compared to days required by the conventional culture method. A faster turnaround time is required for early diagnosis of infection which facilitates an early prescription of an adequate treatment regimen for infected patients. The low rate of detection of infection in culture can be attributed to the fact that the patients with dyspeptic symptoms who were referred for endoscopy might have received antibacterial drugs, which might indirectly interfere with distribution of *H. pylori* in the stomach mucosa by changing the pH of its bacterial niche, leading to its disappearance in the antrum.<sup>2</sup> The other attributing factors for culture negativity are the fastidious, oxygen-sensitive and Patchy nature of H. pylori which may be mainly responsible for our low culture reproducibility of *H. Pylori* from clinical specimens. This finding correlates with a number of studies where the culture method is shown to be less sensitive owing to the above mentioned factors.8-11 However, our finding is in contrast with the findings of Lage et al where they indicated that sensitivity of the culture method is similar to that of PCR of H. pylori urease gene directly from biopsy specimens for diagnosing H. pylori infection.<sup>12</sup> Molecular methods such as PCR are widely used nowadays for accurate diagnosis of *H. pylori* infections directly from gastric biopsy samples since it is less tedious and timeconsuming than culturing of the bacteria.<sup>13-16</sup> This method can also be applied for analysis of diversity of virulence as well as their resistance patterns. Patel SK, et al in their study considered PCR and nested PCR as gold standard molecular techniques which can be easily performed by designing primers extremely specific to H. pylori; as compared to culture method which is considered as disadvantageous owing to its time consuming and less accurate interpretation of results.<sup>17</sup> Wang YK et al. also considered PCR to be a highly sensitive and widely used diagnostic technique which delivers more accurate results of detecting H. pylori in patients with gastro-intestinal bleeding.18

In conclusion, our PCR assay has been shown to be comparatively more sensitive than culture method since it overcame the delays associated with conventional culture methods for detection of infection. Molecular analysis can be regarded as 'diagnostic technique of choice' owing to its accuracy and rapid detection of infection even from small quantities of samples.<sup>11, 13, 19</sup> Therefore PCR directly from biopsy samples can be considered as a very useful diagnostic tool for *H. pylori* infection at times when this gastric bacterium may go undetected by culture or other diagnostic techniques.<sup>20, 21</sup> This study also showed that PCR has a potential value for determining the pathogenicity of *H. pylori* by simultaneous detection of the virulent genes directly from gastric biopsies, thus allowing rapid and accurate determination of high risk patients with gastro-intestinal disease.

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# SOURCE OF SUPPORT

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#### Conflict of Interest: None Declared.

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