Original Article

A Cross-Sectional Study on Molecular Detection of *Helicobacter pylori* cytotoxin-associated gene A and *16SrRNA* Gene from Gastric Biopsy Specimens

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Abstract

Introduction: The aim of the study is relative proportion of cytotoxin-associated gene A *(cagA)* virulence marker in *Helicobacter pylori* isolates and gastric biopsy samples by polymerase chain reaction (PCR). **Methods:** This cross-sectional study was conducted at a tertiary care hospital setting. Gastric biopsy tissues from 200 patients, suffering from upper gastrointestinal tract disorders, were examined for *H. pylori* infection using methods, such as hematoxylin and eosin (H and E) staining, *16S rRNA (Ribosomal ribonucleic acid)*, and *cagA* gene PCR. Chi-square and kappa statistics were used to find the association and agreement between the tests, respectively; $P \leq 0.05$ was considered statistically significant. Screening tests' accuracy was calculated in terms of sensitivity and specificity along with positive and negative predictive values. **Results:** Out of 200 patients, *H. pylori* was detected in 14.5%, 48.5%, and 31% patients by H and E staining, *16S rRNA,* and *cagA* PCR, respectively. Sensitivity and specificity of *cagA* PCR as compared to H and E staining were 89.6% and 78.9%, respectively. **Conclusions:** *CagA* detection directly from biopsy specimen by PCR can potentially and rapidly determine the patient's status, especially when at a higher risk of peptic ulcer.

Keywords: Culture, cytotoxin-associated gene A, diagnosis, gastric biopsy, *Helicobacter pylori,* polymerase chain reaction

Introduction

Helicobacter pylori (H. pylori) is a fastidious, Gram-negative, microaerophilic, spiral-shaped bacterium present in gastric mucosa of the 50% human beings across the world population and 70%–90% of human population in developing countries.[1,2] About 20% of *H. pylori*-infected individuals tend to develop gastrointestinal disorders, during their lifetime.[3]

H. pylori plays a significant role in chronic gastritis and peptic ulcer disease development and is a potential risk factor for mucosa-associated lymphoid tissue lymphoma and gastric adenocarcinoma.[4] The consortium between *H. pylori* and gastroduodenal diseases reveals a necessity for diagnosing the *H. pylori* presence in acid peptic disease.[3] Despite a number of diagnostic methods available for *H. pylori* detection, such as smear examinations, rapid urease test, serological, urea breaths, and fecal antigen tests along with bacteriological culture and its identification, none of the method individually meets the requirement of sensitivity and specificity for its identification.[5] Therefore, a combination of the tests is required to get the highest positive results.[6]

Researchers have made multiple attempts previously to implement molecular diagnostic tests for early diagnosis of *H. pylori* to prevent further complications.[1,7,8] They pointed out that molecular diagnostic tests such as polymerase chain reaction (PCR) are preferable choice due to higher sensitivity and specificity even at low bacterial load.[9,10] Different genes, such as urease A (ureA),[8,11]16S rRNA,[12] *23S rRNA*,[8,9] cytotoxin-associated gene A (*cagA*),[8] and vacuolating toxin A (*vacA),*[8] have been targeted by researchers to improve the

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detection of *H. pylori*. However, c*agA* and v*acA* can determine the clinical consequence of *H. pylori* infections.[8]

cagA gene, a marker of pathogenicity island and indicator of severity due to various manifestation, is not possessed by all strains.[8] Moreover, *cagA* gene polymorphism is evident in *H. pylori* isolates from different geographical regions.[13] Although majority of *H. pylori* isolates from peptic ulcer cases are *cagA*+,[14] there are very few studies directly investigating *cagA* gene presence using stomach biopsy.[13,14] Therefore, we have evaluated PCR technique for detecting *cagA* gene presence in *H. pylori* isolates from both stomach biopsies and their corresponding *H. pylori* culture isolates.

Methods

Study design

This was a cross-sectional study involving gastric mucosal biopsies from 200 subjects with acid peptic disease, which were obtained aseptically during endoscopy.

Sample size calculation

Two hundred gastric tissue biopsies from the study subjects were enrolled. Estimation of sample size was done based on the following formula:

$$N = Z2q / d2p$$

where N = sample size

p = sensitivity *(70%),*
q = 100-*p,*
d = absolute error which is 10%
z-1.96-2

Hence, the sample size is $N = (2)2 \times \frac{30}{0.01} \times 70 = 171N$

$N = 200$

Error = 10%, sensitivity = 70%, 95% confidence interval

Study setting

The study was conducted in the Microbiology department of a tertiary care hospital in accordance with the Declaration of Helsinki and was approved by the local ethics committee of the institute. Written informed consent was obtained from all patients before their enrollment in this study. The data were collected from October 2012 to 2014.

Eligibility criteria

Subjects included in the study were from both the genders, comprised different age groups, suffering from gastric and duodenal diseases only, and having upper gastrointestinal disease complaints, such as upper abdominal pain, vomiting, and epigastric pain, immediately after food, empty stomach, and 2 h after food. However, subjects on analgesics, antibiotics, and proton-pump inhibitors before 48 h of endoscopic procedure and/or having prior history of gastric surgery or active bleeding ulcers were excluded from the study. Informed consent was taken from the subjects before their enrollment into the study.

Endoscopic samples were collected from the overnight-fasted subjects using a fiberoptic endoscope. Endoscope and biopsy forceps were soaked in 2% glutaraldehyde for 30 min[15] before endoscopy and rinsed thoroughly with sterile saline just before the specimen collection. All the biopsy samples were immediately transferred to 10% buffered formalin solution that was used for bacteriological culture using Columbia agar and PCR.

Hematoxylin and eosin staining

Gastric tissue sections of antral part from 10% buffered formalin were processed for hematoxylin and eosin (H and E) staining by following the standard protocol.[16] Identification of *H. pylori* was done under a microscope (×400) by observing pink, curved bacilli on luminal surface of gastric epithelium. H and E staining was taken as a reference method in order to compare other methods.[17]

Molecular characterization of *16S r RNA* and cytotoxin-associated gene A genes

PCR was used to detect *H. pylori* with the help of primers specific for *16S rRNA* and *cagA genes*. *16SrRNA* gene was used for reclassification of the organism and *cagA* gene acts as virulence marker; hence, a combination of genes was used to identify pathogenic *H. pylori*.

Primers for *16S rRNA* and cytotoxin-associated gene A genes

16S rRNA primers

Forward: JW 21:5′-GCGACCTGCTGGAACATTAC-3′ and

Reverse: JW 22: 5′-CGTTAGCTGCATTACTGGAGA-3′

Product size: 139 bp

Cytotoxin-associated gene A primers

cag A Forward: 5′-AGACAACTTGAGCGAGAAAG-3′ and

cag A Reverse: 5′-TATTGGGATTCTTGGAGGCG-3′,

Product size-320 bp

DNA extraction

DNA was extracted from the gastric biopsy tissues and their corresponding bacterial isolates along with reference strains of *H. pylori* – ATCC 13629 and ATCC 43504 (procured from IMTECH Chandigarh) using the modified proteinase K method as per the standard protocol.[12] Deparaffinization of tissue was done before DNA extraction using the protocol of Gohar and Mohammadi.[18] DNA was extracted from both biopsy samples and their corresponding bacterial isolates and amplified using PCR by following the standard protocols.[19] Further, all the samples were kept at −20°C, until further use.

Polymerase chain reaction protocol

Primer and target combination were optimized and the same was used throughout the study. Following DNA extraction, PCR reaction mix was prepared in a sterile microcentrifuge PCR tubes by adding 10 µl master mix, 1.5 µl DNA, 0.5 µl forward primer (10 pM), 0.5 µl reverse primer (10 pM), and

7.5 µl nuclease-free water, to make up the final volume of 20 µl. This was followed by mixing and centrifugation for 5 s at <2000 rpm/min.

PCR reaction mix was amplified using a thermocycler (Veriti, Applied Biosystems, USA), with initial denaturation (1 cycle at 95°C for 5 min), followed by denaturation (35 cycles, 95°C, 1 min), annealing, and extension cycles (55°C and 72°C for 1 min each). Final extension for 1 cycle was performed at 72°C for 10 min. Amplified samples were kept at 4°C until use.

Identification on gel

Electrophoresis of 10 µl amplified product was done using 2% agarose along with standard molecular weight markers, followed by DNA staining with 0.5 µg/ml ethidium bromide solution. The bright fluorescent bands of amplified PCR products (product size – *16S r RNA*: 139 bp and *cag A*: 320 bp) and 100 base pair DNA molecular marker were visualized under ultraviolet light using Syngene G Box image analysis system.

Statistics

SPSS software version 12 (Chicago, USA) was used for the data analysis. Sensitivity and specificity along with positive and negative predictive values were calculated to assess the accuracy for screening tests. Chi-square test and kappa statistics were applied to find association and agreement between the tests, respectively. $P \leq 0.05$ was considered statistically significant.

Results

As per the demographic distribution of the subjects, 50.5% belonged to the age group of <40 years, while 20.5%, 17.5%, and 11.5% were from the age group of 51–60 years, 41–50 years, and >61 years, respectively. Male predominance was observed to be 76%. *H. pylori* was detected in biopsy samples by observing the pink, curved bacilli on luminal surface of gastric epithelium (×400) using H and E method, as presented in Figure 1.

Out of 200 subjects, 29 were *H. pylori*-positive cases identified using H and E method, out of which 21 and 8 were cases of chronic gastritis and peptic ulcer, respectively. However, only five subjects were found to be positive for cultures, out of which two had chronic gastritis and three suffered from peptic ulcer [Table 1]. Representative images of gene identification on gel of *16S rRNA* and *cagA* genes are presented in Figures 2 and 3, respectively. *16S rRNA* PCR detected 97 positive *H. pylori* cases, out of which 87 were chronic gastritis and 10 were peptic ulcer cases, whereas *cagA* PCR identified 62 *H. pylori* positive cases, of which 53 cases were chronic gastritis and 9 were peptic ulcer, respectively. All the methods were found to be statistically significant ($P < 0.05$) in evaluating the *H. pylori*-positive cases, as tabulated in Table 1. Moreover, all the positive cultures and their corresponding gastric biopsy samples showed positive results for *cagA* gene and *16S rRNA* by PCR.

The comparative account of *16S rRNA* PCR and *cag A* PCR showed 48.5% and 31% positive cases, respectively, when compared with H and E staining, which yielded 14.5% positive cases [Table 2]. However, the sensitivity and specificity of *16S rRNA* PCR were found to be 96.55% and 59.65%, respectively, while *cag A* PCR sensitivity and specificity were found to be 89.66% and 78.95%, on comparing with H and E staining, respectively [Table 3]. The kappa values were found to be 0.2848 and 0.4659, thus showing moderate agreement on comparing the H and E staining results with *16S rRNA* PCR and *cagA* PCR results, respectively [Table 3].

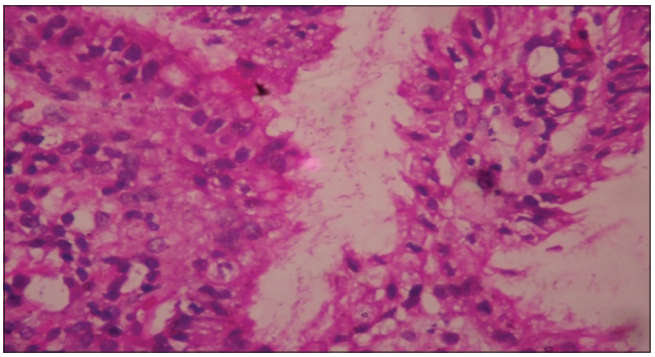

Figure 1: Hematoxylin and eosin staining showing curved bacilli over luminal surface of gastric epithelium (×400)

Figure 2: Gel electrophoresis of the polymerase chain reaction amplified product of *Helicobacter pylori16S rRNA*

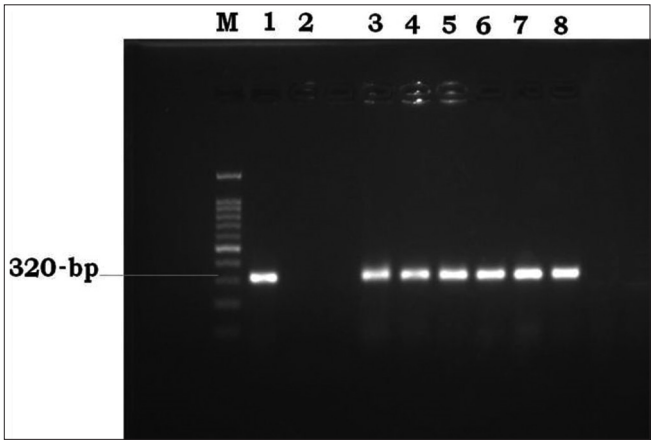

Figure 3: Gel electrophoresis of *Helicobacter pylori* cytotoxin-associated gene A gene

Table 1: Comparison of endoscopic diagnosis

Methods	Status	Chronic gastritis (%)	Peptic ulcer (%)	Total
H and E	Positive	21 (72.41)	8 (27.59)	29
	Negative	169 (98.83)	2 (1.17)	171
	Chi-square with Yates's correction, *P*		31.0783, 0.0001	
Culture	Positive	2 (40.00)	3 (60.00)	5
	Negative	188 (96.41)	7 (3.59)	195
	Chi-square with Yates's correction, *P*		21.8627, 0.0001	
16S rRNA	Positive	87 (89.69)	10 (10.31)	97
	Negative	103 (100.00)	0 (0.00)	103
	Chi-square with Yates's correction, *P*		9.1126, 0.0030	
cag A	Positive	53 (85.48)	9 (14.52)	62
	Negative	137 (99.28)	1 (0.72)	138
	Chi-square with Yates's correction, *P*		14.3508, 0.0001	

rRNA: Ribosomal ribonucleic acid, H and E: Hematoxylin and eosin

Table 2: Comparative account of hematoxylin and eosin stain versus 16S ribosomal ribonucleic acid polymerase chain reaction and hematoxylin and eosin stain versus cagA polymerase chain reaction

PCR	H and E stain versus 16S rRNA PCR			H and E stain versus cagA PCR		
	H and E stain			H and E stain		
	Positive (%)	Negative (%)	Total (%)	Positive (%)	Negative (%)	Total (%)
Positive	28	69	97 (48.5)	26	36	62 (31.0)
Negative	1	102	103 (51.5)	3	135	138 (69.0)
Total	29 (14.50)	171 (85.50)	200 (100.0)	29 (14.50)	171 (85.50)	200 (100.0)

rRNA: Ribosomal ribonucleic acid, H and E: Hematoxylin and eosin, PCR: Polymerase chain reaction

Table 3: Statistics of diagnostic tests in terms of sensitivity, specificity and κ values

Comparison of H and E stain versus 16S rRNA PCR and H and E stain versus cagA PCR		
Statistics	**H and E stain versus 16S r RNA PCR (%)**	**H and E stain versus cagA PCR (%)**
Sensitivity and specificity		
Sensitivity	96.55	89.66
Specificity	59.65	78.95
Positive predictive value	28.87	41.94
Negative predictive value	99.03	97.83
κ statics		
Agreement of test	65.00	80.50
Desired agreement	51.07	63.49
κ	0.2848	0.4659
P	<0.01*	<0.01*

*Represents $P<0.05$. rRNA: Ribosomal ribonucleic acid, H and E: Hematoxylin and eosin, PCR: Polymerase chain reaction

Discussion

In this study, H and E staining was taken as reference method as it is a popular and practical diagnostic method[20] with an added advantage of providing histopathologic features of stomach epithelium, such as degree of inflammation and mucosal changes[21] and compared with PCR results of *16S rRNA* and *cagA* gene to calculate accuracy in terms of sensitivity, specificity, and predictive values (positive and negative).[17] Similar approach of *H. pylori* detection using H and E staining was done by Mehmood *et al*.[22]

H. pylori is usually seen in active chronic gastritis due to prolonged colonization and inflammation of epithelial lining of stomach in most of the individuals.[23] Conversely, only 2% of patients were noticed with minimal or no mucosal inflammation when suffering from *H. pylori* infection in the present study.

Our observation showed 14.5% positivity with H and E staining of gastric tissue sections; however, different degrees of positivity were reported by researchers, which were not accorded with our results.[5,21,23,24] Adlekha *et al*.[24] found that H and E staining resulted in 62% of positive cases out of 530 specimens collected from gastric endoscopic examinations. Whereas, Akanda *et al*.[5] and Ahmad *et al*.[23] reported 45.6% and 34% positive cases by H and E staining,

respectively. On the contrary, Kaur *et al*.[21] reported 8% prevalence on histological examinations of biopsies.

Furthermore, sensitivity and specificity of the histology were reported to be affected due to various reasons: expertise or trained personnel and the levels at which the sections were taken may affect the positivity of the H and E staining. For example, in some cases of gastric atrophy where the bacterial density is less because of deprivation of nutrients, resulting in less chance of *H. pylori*-positive cases from biopsies. These changes led to decreased stomach bacterial load, owing to scarcity of nutrients for *H. pylori*[25] and may lead to false negative test by H and E method.[26]

We used JW 21 and 22 primers,[27] which showed specificity for *H. pylori* DNA detection by the presence of a 139-bp fragment of *16S rRNA* gene and was in accordance with the findings of Samra *et al*.[28] where they detected 139-bp fragment in 10 biopsies samples out of 18. They detected an amplified 16S *rRNA* gene segment of 139 bp from drinking water and vomiting fluids, thereby confirming that direct detection of *H. pylori* positive samples can be possible by PCR. Moreover, PCR specificity is useful in ruling out the cross-reaction with other bacteria, such as *E. coli* and other important organism, such as *Campylobacter* and *Helicobacters* (*H. cinnaedi*, *H. fenelliae*, and *H. muselae*) species.[29] Recent studies showed that PCR analysis had improved sensitivity to detect DNA, encoded from *16S rRNA* gene of *H. pylori* from gastric tissue samples and possessed a higher degree of positivity and accuracy (55% and 80%)[20] to demonstrate *H. pylori* presence[30,31] as compared to H and E method. However, variations in PCR detection rates could be possible due to low bacterial load in biopsies.

Till date, only few studies had analyzed such a large sample number and our study was among one of them where we studied 200 subjects and analyzed the biopsy samples by PCR for *16S rRNA* and *cagA* genes. We observed that 97/200 (48.50%) cases were positive for *16S rRNA* PCR with 96.55% sensitivity and 59.65% specificity. Hence, our observations showed the importance of PCR for *H. pylori* detection from stomach biopsies. Smith *et al*.[32] reported that 52.38% of cases were found to be of *H. pylori* positive with 100% sensitivity and 68% specificity for *16S rRNA* on comparing with H *and* E staining. In western regions 60%-70% of *H. pylori* strains were cagA positive and were associated with gastric cancer and duodenal ulcer.[33] Thus, we also detected the *cagA* presence by PCR, a pathogenicity indicator using gastric biopsies and 62 (31%) subjects were *cagA* positive with 89.66% sensitivity and 78.95% specificity. Similar results were reported by Smith *et al*.[32] detection of 35% *cagA* positive (19/42) of the biopsy specimens of *H. pylori* were among Nigerian people with 92.9% sensitivity and 78.6% specificity on comparing with histological examinations (H and E staining and Giemsa staining). Conversely, higher occurrence of *cagA* was reported by Bindayna *et al*.[34] in biopsy samples (59%) and clinical isolates (62%).

The present study also demonstrated the existence of a good association of cag*A*+ strains between biopsies with endoscopic findings, which correlates with observations of Bindayna *et al*.[34] Our finding of 85.48% subjects of chronic gastritis and 14.52% of peptic ulcer pointed toward the important findings of *cagA* gene positivity among different clinical conditions suffering from gastric problems and were in accordance with the results of Bindayna *et al*.[34] having *cagA* positivity of 80% and 100% for duodenal ulcer and chronic gastritis cases, respectively. Therefore, *cagA* presence may be associated with increased manifestations of complications. Thus, the presence of certain genotype, such as *cagA* in isolates, can be closely related to cause severe inflammation of stomach mucosa, leading to severe gastritis and peptic ulcer.[8]

Furthermore, five *H. pylori* positive cultures were isolated out of 200 biopsy samples that were grown on culture media. All the five cultures along with their corresponding gastric biopsy samples were tested and noted to be 100% *cagA* positive in accordance with the results of Mishra *et al*.[12] where 54 bacterial isolates along with their respective biopsy tissues were *cagA* positive, out of which majority of patients suffered from duodenal ulcer[18] and chronic gastritis.[23] Furthermore, *cagA* gene identification by PCR directly from samples of gastric biopsy carries more value as *H. pylori* is microaerophilic and fastidious organism and its culturing is difficult, which takes longer time for culturing and identification. Therefore, *cagA* gene typing helps in identifying strains that are associated with pathogenicity and disease severity. However, geographical, environmental, and agent determinants together determine disease outcome.

Thus, *H. pylori* diagnosis using PCR can detect *H. pylori* DNA that can act as an indicator of infections. Importance of PCR is due to its specificity, rapid test outcome, and detecting DNA from nonviable cells. However, every method has certain disadvantages and the major disadvantages of PCR include practicability and cost factor along with false negativity due to the presence of inhibitors, thereby affecting the result of PCR.[35,36] Our observation on PCR for *16S rRNA* gene detection from biopsies was found to be quite sensitive, thereby establishing its high prognostic value where it can detect bacteria even at very low number that were usually missed by other methods, including culture. Furthermore, genotypic level variability exhibited by *H. pylori* strains has got importance in the molecular biology research field. Combination of *16S rRNA* and *cagA* PCR can detect *H. pylori*, belonging to virulent species, owing to its specificity, thereby confirming the presence of pathogenic *H. pylori*. Hence, our study showed the importance of PCR for studying *16S rRNA* and c*agA* directly from gastric biopsies, thereby allowing rapid detection of patients who are prone to develop peptic ulcer and infection detection at early stage is crucial for preventing further complications. In future, this study can be extended to other virulence-associated genes.

Conclusion

Detection of cagA directly from biopsy specimen helps in rapid diagnosis of H pylori infections.

Research quality and ethics statement

This study was approved by the Institutional Review Board/ Ethics Committee of K.L.E. University, Belgaum, India. IRB No 2494 (A) 2012-13. The authors followed applicable EQUATOR Network ("http:// www.equator-network.org/) guidelines during the conduct of this research project.

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Conflicts of interest

There are no conflicts of interest.

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