"MOLECULAR CHARACTERISATION OF HELICOBACTER PYLORI FROM GASTRIC BIOPSY SPECIMENS IN ACID PEPTIC DISEASE"

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For the Award of the Degree of
DOCTOR OF PHILOSOPHY

In the Faculty of
MEDICINE

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INTRODUCTION

*Helicobacter pylori (H pylori)* is a fastidious, gram-negative, microaerophilic and spiral shaped bacterium present in the human gastric mucosa of 50% of the world population and 70-90% of the population in developing countries.\(^1,2\) The association between *H pylori* and gastroduodenal diseases demonstrates the need to diagnose the presence of bacteria in acid peptic disease.\(^3\)

Culturing of *H. pylori* is both difficult and time-consuming but 100% specific, so many clinical laboratories are attempting to implement molecular diagnostic tests\(^1,4\) for early diagnosis and prevention of complications. Therefore it is important to detect the presence of *H pylori* in gastroduodenal diseases, evaluate the sensitivity of culture technique for diagnosis of *H pylori* and look for virulence mechanisms.

The *cagA* gene which is the pathogenicity island marker may not be present in every strain of *H pylori*. The association of severity of clinical outcomes is determined by the presence of *cagA* gene.\(^5\) However there are very few studies looking directly at the presence of *cagA* gene in gastric antral biopsies of patients.\(^6,7\) Therefore the present study is done to evaluate PCR for detection of *H pylori cagA* gene in gastric biopsies as well as their corresponding *H pylori* isolates.

The resistance of *H pylori* to the antimicrobials is a growing problem. Therapeutic regimens followed currently are mostly based on either insufficient data or obtained from other geographically unrelated regions.\(^8\) This study was done to evaluate the antimicrobial resistance pattern in this region.
OBJECTIVES

• To study the relative proportion of cagA pathogenicity marker from Helicobacter pylori isolated strains & their respective gastric tissue biopsies by molecular method like PCR.

• To evaluate culture technique in identification of Helicobacter pylori on the gastric tissue biopsies.

• To generate baseline data on antibiotic susceptibility pattern.
REVIEW OF LITERATURE

Warren and Marshall stated in their original paper that gastritis and stomach ulcers were caused by bacterial infection and not associated with consumption of spicy food or by stress as thought before. By 1984 it had become clear that *H pylori* infection was strongly associated with chronic gastritis. Marshall and Warren established the role of *H pylori* in peptic ulcer. In 1994 the international agency for cancer research, an arm of the world health organisation declared that *H pylori* had been classified as group 1 or definite human carcinogen. Persistent infection with *H pylori* is a risk factor for the development of gastric adenocarcinoma especially of the distal stomach.

The analysis of sequence of 16S rRNA gene led to differentiation of *Helicobacter pylori* from *Campylobacter* species. Other important features that distinguished *Helicobacter* species from *Campylobacter* species included the possession of sheathed flagella, unique fatty acid profile, active urease enzymes and a distinct protein profile.

Fecal-oral transmission is most important. *H pylori* has been isolated from the feces of young children infected with the organism. It is estimated that *H pylori* positive patients have a 10-20% lifetime risk of developing ulcer disease and a 1-2% risk of developing distal gastric cancer. The risk of development of these disorders in the presence of *H pylori* infection depends on a variety of bacterial, host and environmental factors that relate to the pattern and severity of gastritis.

*H pylori* requires for growth a nutrient-rich medium, serum and a microaerobic environment. The organism requires high humidity (98%) and
Review of Literature

A microaerophilic environment (10% CO₂, 15% O₂ and 85% N₂) is required for growth. Growth occurs at optimum temperature of 37°C and neutral pH. H. pylori is a fastidious microorganism and requires complex growth media. These media are supplemented with blood, haemin, serum, starch or charcoal. These supplements protect against the toxic effects of long-chain fatty acids. Commonly used solid media for isolation and culture consist of Columbia agar or brucella agar supplemented with sheep or horse blood or fetal calf serum. Growth is seen on incubation at 35°C-37°C for 7-10 days. Positive cultures are usually detected after 3-5 days in primary cultures and in 2-3 days in subsequent subcultures. The colonies are small, 0.5-1.0 mm in diameter, translucent, convex and smooth colonies. The organism shows great degree of oxygen sensitivity. Subcultures done more than two hours on bench are sometimes unsuccessful. Peptone water or nutrient broth are not suitable for growth of H. pylori.

Strain-specific H. pylori gene, cytotoxin-associated gene A (cagA) was identified in 1989. CagA gene has been recognized as a pathogenicity marker for H. pylori strains that constitute increased risk for peptic ulcer disease and gastric cancer. CagA is a marker for the 35-40kb cag pathogenicity island which contains 31 genes encoding bacterial type IV secretion system. One substrate for the type IV system in H. pylori is the cagA product which is injected into epithelial cells. CagA undergoes tyrosine phosphorylation by a host cell kinase and the new phosphoprotein alters the physiology of the affected cells including apoptosis and cell morphology. 50-70% strains of H. pylori possess CagA. Higher inflammatory response is seen in patients infected with cagA+ strains and are at increased risk for developing symptomatic outcome (peptic ulcer or gastric cancer).
*H pylori* infection is diagnosed by invasive and non-invasive methods. Invasive method i.e endoscopic biopsy based methods include bacteriologic culture, histopathologic studies, smear examination with Gram’s and Giemsa stain, rapid urease test and molecular studies. Non-invasive methods include serologic testing, antigen detection in the stool and urea breath test.

*H pylori* growth is possible in a candle jar. It results in small colonies and takes longer time. The optimal culture temperature is 37°C. For primary culture colonies may appear after 3 days. In the case of negative culture 7-10 day incubation is recommended to ensure that the result is negative. Subcultures take 2-3 days to grow. Typical curved bacilli on Gram’s staining with presence of catalase, oxidase and urease activity from the cultured bacteria is identified.

Molecular methods are widely used for the diagnosis of *H pylori* infection as well as analysis of diversity, virulence, persistence and resistance patterns of these bacteria. Its application includes not only in the detection of the bacterium but also the specific genes relevant to pathogenesis like *cagA* and the specific mutations associated with antimicrobial resistance. Standard PCR for detection of *H pylori* and Standard PCR for detection of pathogenic factors of *H pylori* like *cagA* and *vacA* gene. Strains harbouring the *cagA* are associated with more severe diseases especially peptic ulcer disease and gastric adenocarcinoma as well as precancerous lesions.

In 70-90% of cases the treatment is successful but the presence of antimicrobial-resistant strains of *H pylori* may result in treatment failure. Various in vitro antibiotic susceptibility methods have been used: agar dilution, E test and disk diffusion. The prevalence of Clarithromycin, Metronidazole and Amoxicillin
resistances varies between countries, regions and is highest for Metronidazole. Tetracycline and Ciprofloxacin resistance has been reported but uncommon.\textsuperscript{24}
MATERIAL AND METHODS

Source of Data:

Gastric (antral) mucosal biopsies, obtained during endoscopy in the Gastroenterology department of Dr Prabhakar Kore, KLE’S Hospital and Medical research centre from patients with acid peptic disease.

Study Design: Hospital based descriptive study.

Method of Collection of Data

This study was conducted in the Department of Microbiology, KLE University’s J N Medical college, Belagavi from October 2012 to October 2014 with the support of Departments of Pathology, Gastroenterology and KLE University’s Dr Prabhakar Kore, Basic Science Research centre Belagavi. The study group consisted of 200 patients varying in different age and sex who were referred for endoscopy.

Inclusion criteria:

• Subjects with upper gastro intestinal disease complaints.

• Subjects with gastric & duodenal diseases.

Exclusion criteria:

• The patients who have received NSAID’S, antibiotics and proton pump inhibitors 24-48 hrs prior to endoscopy.

• Patients who have history of prior gastric surgery.

• Patients with active bleeding ulcers.
Sample Size (n) Estimation:

200 gastric biopsy samples were collected from patients with acid peptic disease.

Estimation of sample size was based on using the formula

\[ N = \frac{z^2 q d^2}{p} \]

where

- \( N \) = sample size
- \( p \) = sensitivity (70%)
- \( q = 100 - p \)
- \( d \) = absolute error which is 10%
- \( z \) = 1.96 (for 95% confidence interval)

\[ N = 171 \]

Hence, sample size is

\[ N = 200 \]

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

Ethical clearance: The study was conducted with the approval from the KLE University’s ethics committee on human subjects. Informed consent was obtained from the patients before the enrolment into the study.

Sample collection and transportation:

Biopsy Sample: Five biopsy samples were taken from the antrum (2cm from the pylorus) and were transferred to respective Eppendorf tube under sterile conditions. One sample was inoculated into urea broth for rapid urease, two specimens were
transported in brain heart infusion broth with 10% glycerol for culture, Gram’s stain and Giemsa stain. Specimens were placed in 10% buffered formalin for histopathological examination and PCR. The specimens for culture were transported in ice to the laboratory and were inoculated on to the culture media without delay.

**Sample processing:**

**Rapid Urease Test**

Biopsy tissue was inoculated in an Eppendorf tube containing 0.5 ml of unbuffered 10% urea solution in de ionized water at pH of 6.8 to which had been added two drops of 1% phenol red as a pH indicator. An uninoculated tube was also kept as a negative control. The hydrolysis of the urea liberated ammonia which increased the pH and changed the colour from yellow to pink at room temperature within 30 minutes thus indicating a positive test.\(^9\)\(^{25}\)

**Culture:**

The biopsy tissue was crushed between two sterile glass slides and the minced tissue was inoculated onto Columbia blood agar with 10% sheep blood with campylobacter growth supplement and campylobacter selective supplement, incubated in CO\(_2\) jar with multiple wax candles lighted to create the micro-aerophilic atmosphere. While closing the jar each time, petroleum jelly was put on the side of the rim of the jar and then the jar was closed tightly. The jar was kept at 37\(^\circ\)C for 5-7 days.\(^{26}\) All plates showing small, circular, translucent and gray colonies were processed for identification. The bacterial colonies were identified on the basis of colony morphology, Gram’s staining, positive oxidase, catalase and urease reactions. Gram’s stain was performed on all suspected growths. The smears were scanned.
under oil immersion objective (X 1000) of a light microscope and observed for gram negative, long curved bacilli.

**Microscopic examination of the smears:**

Another biopsy tissue was crushed between two sterile glass slides and the minced tissue was used to make two smears for Gram’s and Giemsa staining. The slide was counter stained with dilute carbol fuschin and observed for typical curved/spiral/sea gull shaped Gram negative bacilli with bluntly rounded ends under oil immersion (X1000) objective of a light microscope. The modified Giemsa stain was done for another slide smear. Typical blue colored, curved/spiral/sea gull shaped bacilli were observed under oil immersion (X1000) objective of a light microscope. The slides were stained with Haematoxylin & Eosin (H&E) stain. The curved rods on the luminal surface of the gastric epithelial cells were identified as *H pylori* (X400).

**Antibiotic Susceptibility Testing of the Isolates**

A Total of 05 isolates of *H pylori* subcultured were tested for antibiotic susceptibility using modified Kirby-Bauer disc diffusion method on Mueller-Hinton agar plate supplemented with 10% sheep blood. Standard inoculums of *H pylori* culture was suspended in BHI broth. The turbidity was adjusted equal to Mc Farland 3. The inoculums was seeded on to Mueller Hinton blood agar plate using sterile cotton wool swab, antibiotic discs with the following drug contents: metronidazole(5μg), amoxicillin(10μg), tetracycline(30μg), erythromycin(15μg), levofloxacin(5μg), norfloxacin(5μg) and cotrimoxazole(10μg) were placed on the plates. The plates were incubated at 37°C in CO₂ jar for 3-4 days. The results were interpreted as per clinical laboratory standards institute (CLSI) 2011 guidelines.
ATCC 26695 strain of *H. pylori* was used as a reference strain for antibiotic susceptibility testing.\(^\text{31,24}\)

**Detection of *H. pylori* Infection by Polymerase Chain Reaction (PCR)**

PCR was used to detect *Helicobacter pylori* by using primers specific for 16S rRNA gene. 16S rRNA gene is targeted to confirm *H pylori* infection and the presence of the pathogen is confirmed by the amplification of *H pylori* specific DNA.\(^\text{32}\)

**Primers:**

16S rRNA: Forward: JW 21- 5\(^{1}\)GCGACCTGCTGGAACATTAC3’

16S rRNA: Reverse: JW 22-5\(^{1}\)-CGTTAGCTGCATTACTGGAGA3’


Product size-139 bp

**Methodology:**

**Deparaffinisation of Tissue**

**Deparaffinization Method**

1. Deparaffinization of the Tissue sections was done with 500 microliter 100\% xylene under a fume hood. Then contents were vortexed for 1 minute and left in a 65 °C water bath for 15 minutes.

2. The procedure was repeated two more times with decantation of xylene solvent.
Material and Methods

Xylene Removal

The samples were washed five times with Ethanol to remove the residual xylene as follows.

1. 1ml of absolute ethanol was added and mixed by vortex for ten seconds and removed after 10 minutes.
2. 1ml of absolute ethanol was added and mixed by vortex for ten seconds and removed after 30 minutes.
3. 1ml of 90% ethanol was added and mixed by vortex for ten seconds and after 20 minutes, tube was centrifuged at 8000 RPM and then removed ethanol out.
4. 1ml of 70% ethanol was added and mixed by vortex for ten seconds and after 20 minutes, tube was centrifuged at 8000 RPM and then removed ethanol out.
5. 1ml of 50% ethanol was added and mixed by vortex for ten seconds and after 20 minutes, tube was centrifuged at 8000 RPM and then removed ethanol out.

The microtubes were then left in a 40º C oven to dry the tissues.

DNA Extraction

1. Vortex the sample to dislodge into the TE buffer medium and Centrifuge at 12000 rpm for 10 min.
2. Discard the supernatant. Add 500µl fresh T.E. buffer, vertex for 3-4 seconds, centrifuge for 12000 rpm for 10 minutes
3. Discard supernatant, add 500 µl of T.E. buffer, vertex for 3-4 sec, centrifuge at 12000 rpm for 10 min
4. Discard supernatant, add 50 µl lysis buffer I, Vortex for 3 sec
5. Centrifuge at 10000 rpm for 5 min
6. Add 50 µl Lysis buffer II, vertex for 3 sec, centrifuge at 10000 rpm for 5 min
Material and Methods

7. Add 10 µl Proteinase-K (100ug/ml), vortex for 3 sec
8. incubate in water bath for 2 hrs at 60°C and at 90°C for 10 min
9. Remove from water bath and keep at room temperature for 10-15 min
10. Add 130 µl of 70% ethanol and 10 µl of 3 M sodium acetate, vortex for 5 min
11. Keep at -20°C for overnight
12. Keep at room temperature for 10 min, centrifuge at 12000 rpm for 10 min
13. Discard supernatant, add 100 µl of TE buffer, vertex it, centrifuge at 12000 rpm for 10 min
14. Collect the supernatant in new fresh 0.5 ml centrifuge tube
15. DNA stored at -20°C

PCR Amplification

Table 1: PCR Reaction Mix (20µl volume)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Amount added IX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Master Mix</td>
<td>10 µl</td>
</tr>
<tr>
<td>Forward Primer (5picomol)</td>
<td>1µl</td>
</tr>
<tr>
<td>Reverse primer (5picomol)</td>
<td>1µl</td>
</tr>
<tr>
<td>Nuclear free water to a final volume upto</td>
<td>6.5µl</td>
</tr>
<tr>
<td>Template DNA(75 nanogram)</td>
<td>1.5µl</td>
</tr>
</tbody>
</table>
Table 2: PCR Programming

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95 °C</td>
<td>5 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Final Denaturation</td>
<td>95 °C</td>
<td>60 seconds</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>55 °C</td>
<td>60 seconds</td>
<td>35 cycles</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>60 seconds</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 °C</td>
<td>10 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Soak</td>
<td>4 °C</td>
<td>Indefinite</td>
<td></td>
</tr>
</tbody>
</table>

Procedure:

1. Combine the components as listed in Table 1, except DNA to prepare a master mix in a sterile micro centrifuge.

2. Add master mix in the PCR tubes and Add 1.5µl DNA, mix it well and centrifuge for 5 sec.

3. Place the reaction mixture in a thermal cycler. Start the thermal cycling program.

4. Cycling program is given in the table 2. Each primer / target combination requires optimization.

5. Store the PCR product at -20°C until needed.
Material and Methods

Agarose Electrophoresis

Preparation and Running of Agarose Gel

1. Agarose Powder mixed with 1X electrophoresis buffer is heated in a microwave oven until completely melted. Cool the solution to about 60°C and add 1µl of ethidium bromide (0.5 µg/ml), pour in to casting tray and allow solidifying at room temperature.

2. After the gel solidifies, remove the comb carefully and mount the gel in the electrophoresis tank and cover with electrophoresis buffer.

3. Load the 10µl amplified product into wells and also load 100 bp ladder as marker.

4. Apply the current with fixed voltage (100-150V)

5. The distance DNA has migrated to be judged by migration of tracking dyes. Bromophenol blue migrates through agarose gels approximately 2.2 fold faster than xylene cyanol independent of agarose concentration.

6. After electrophoresis, DNA can be visualized by placing the gel in Gel Documentation system. The gel was visualized using a UV transilluminator and take the photo for further analysis.

7. The amplified PCR products and 100 base pair DNA molecular markers were seen as bright fluorescent bands.

Virulence Marker Detection (cagA gene)

DNA was extracted from the bacterial growth using the same procedure mentioned above as per the standard protocol. The extracted DNA from gastric biopsy tissues and from bacterial growth was subjected to PCR for the presence of cagA.
Material and Methods

Primers

CAG A: Forward: 5'AGACAACTTGAGCGAGAAAG-3

CAG A: Reverse: 5'TATTGGGATTCTTGGAGGCG-3


Product size-320 bp

PCR Amplification- Table 3: PCR Reaction Mix (20µl volume)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Amount added IX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Master Mix</td>
<td>10 µl</td>
</tr>
<tr>
<td>Forward Primer (10 picomol)</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Reverse primer(10 picomol)</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Nuclear free water to a final volume upto</td>
<td>7.5µl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1.5µl</td>
</tr>
</tbody>
</table>

Table 4: PCR Programming

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95 °C</td>
<td>5 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Final Denaturation</td>
<td>95 °C</td>
<td>60 seconds</td>
<td>35 cycles</td>
</tr>
<tr>
<td>Annealing</td>
<td>52 °C</td>
<td>60 seconds</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>60 seconds</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 °C</td>
<td>10 minutes</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Soak</td>
<td>4 °C</td>
<td>Indefinite</td>
<td></td>
</tr>
</tbody>
</table>
Material and Methods

All the PCR reactions were performed using thermocycler (Veriti, Applied Biosystems). The amplified products were electrophoresed in 2% agarose along with standard molecular weight markers, stained with 0.5 µg/ml ethidium bromide solution, visualized by placing the gel in gel documentation system.

Statistical Methods

Sensitivity, specificity, positive and negative predictive value were calculated for screening tests. Chi-square test has been used to find the significant association of the findings. Kappa statistics was used to find the agreement between the tests. P value of ≤0.05 was considered statistically significant. SPSS software version 12 (Statistical package for Social Science, Chicago, USA) was used.
RESULTS

This study was conducted in the department of Microbiology, KLE University’s J N Medical college, Belagavi from October 2012 to October 2014. Two hundred patients who presented with gastroduodenal diseases were included in the study.

Out of 200 study subjects, 50% of study subjects were in the age group of less than 40 years and 20.50 % were in the age group of 51-60 years. 76% were males and 24 % were females. Male to female ratio is 3.12: 1. 50% of the patients were in the age group <40 years and male and female distribution remained same. 20.50% of the patients were in the age group 51-60 yrs, male and female distribution remained same. Females are younger than males by 2 years (mean age). 95% of the patients had chronic gastritis and 5% of the patients had peptic ulcer according to endoscopic diagnosis. Percentage of chronic gastritis is ranging from 94-100% and peptic ulcer is ranging from 6-4.88% clinically. Chronic gastritis is more in older people and peptic ulcer is more in younger people but statistically non-significant since P value >0.8. There is no difference observed between males and females in association with endoscopic diagnosis clinically as well as statistically since P value is >0.05.

Out of 200 study subjects, 18.5% of samples were positive by Gram’s staining method, 29.5% were positive by Modified Giemsa staining, 26.5% were positive by urease test, 2.5% were positive by culture, 48.5% were positive by PCR for detection of 16S rRNA, 31% were positive by PCR for detection of cagA gene and 14.5% were positive by H&E staining. H&E staining is taken as the gold standard according to CDC and compared with all other methods and calculated sensitivity, specificity, positive predictive value and negative predictive values.
Table 5: Statistical analysis of various diagnostic tests for detection of *H pylori*

<table>
<thead>
<tr>
<th>Test</th>
<th>sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Positive predictive value (%)</th>
<th>Negative predictive value (%)</th>
<th>Youden’s Index</th>
<th>Diagnostic effectiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram’s stain</td>
<td>62.075</td>
<td>88.89</td>
<td>48.65</td>
<td>93.25</td>
<td>0.5</td>
<td>85%</td>
</tr>
<tr>
<td>Giemsa stain</td>
<td>75.86</td>
<td>78.36</td>
<td>37.29</td>
<td>95.04</td>
<td>0.54</td>
<td>78%</td>
</tr>
<tr>
<td>Urease test</td>
<td>79.31</td>
<td>82.46</td>
<td>43.40</td>
<td>95.92</td>
<td>0.61</td>
<td>82%</td>
</tr>
<tr>
<td>Culture</td>
<td>13.8</td>
<td>99.4</td>
<td>80</td>
<td>87.2</td>
<td>0.132</td>
<td>80%</td>
</tr>
<tr>
<td>PCR-16S rRNA</td>
<td>96.5</td>
<td>59.6</td>
<td>28.8</td>
<td>99</td>
<td>0.56</td>
<td>65%</td>
</tr>
<tr>
<td>PCR-cagA</td>
<td>89.6</td>
<td>78.9</td>
<td>41.9</td>
<td>97.8</td>
<td>0.68</td>
<td>81%</td>
</tr>
</tbody>
</table>

The 16S rRNA gene PCR assay detected the presence of a 139-bp fragment of the *H pylori* 16S rRNA gene in DNA present in formalin-fixed paraffin embedded biopsy specimens following deparaffinization and solubilization of total nucleic acids. The target sequence was amplified by PCR using primers JW21 and JW22 and detected by the use of UV transilluminator. The assay is specific for *H pylori* only and does not detect *H. cinnaedi, H. fenelliae, H. mustelae,* or various *Campylobacter* species.

Out of 200 gastric biopsy samples 62 were positive by PCR for *cagA*, 29 were positive by H&E stain where 26 are true positives and 135 are true negatives, 03 are
false negatives and 36 are false positives. Thus sensitivity is 89.66%, specificity is 78.95%, positive predictive value is 41.94% and negative predictive value is 97.83%. There is agreement between both methods by weighted kappa statistics.

Out of 200 gastric biopsy samples, *H pylori* was isolated in 05 cultures. All these 5 *H pylori* isolates were positive for *cagA* by PCR and their corresponding gastric biopsy samples were also positive for *cagA* by PCR.

**Fig: PCR Amplification Product of *H Pylori CagA* gene**

Lanes 3,5,7- from Biopsies and 4,6,8-from their corresponding isolates respectively, 1-positive control *Helicobacter pylori* ATCC 43504, 2– negative control and M-Molecular weight marker(100bp)
Table 06: Primary and combined antibiotic susceptibility pattern of *H pylori* isolates (n=05)

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Resistant (No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metronidazole</td>
<td>05</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>03</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>01</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>01</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>01</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>03</td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>01</td>
</tr>
<tr>
<td>Metronidazole +amoxicillin</td>
<td>03</td>
</tr>
<tr>
<td>Metronidazole +amoxicillin+norfloxacin</td>
<td>03</td>
</tr>
</tbody>
</table>

In our study all the five strains were resistant to Metronidazole. Three strains were resistant to Amoxicillin and Norfloxacin. One each resistant to Tetracyclin, Levofloxacin, Cotrimoxazole and Erythromycin. A total of 03 strains were multidrug resistant. They showed resistance to Metronidazole, Amoxicillin and Norfloxacin.
Table 07: Antibiotic susceptibility pattern of *H pylori* isolates (N=05) in relation to gastroduodenal disorders

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Endoscopic diagnosis</th>
<th>Organism isolated</th>
<th>Antibiotic susceptibility pattern of <em>H pylori</em> isolates (N=05)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MTZ</td>
</tr>
<tr>
<td>72</td>
<td>Duodenal ulcer</td>
<td><em>H pylori</em></td>
<td>R</td>
</tr>
<tr>
<td>75</td>
<td>Duodenal ulcer</td>
<td><em>H pylori</em></td>
<td>R</td>
</tr>
<tr>
<td>99</td>
<td>Chronic gastritis</td>
<td><em>H pylori</em></td>
<td>R</td>
</tr>
<tr>
<td>164</td>
<td>Duodenal ulcer</td>
<td><em>H pylori</em></td>
<td>R</td>
</tr>
<tr>
<td>200</td>
<td>Gastric ulcer</td>
<td><em>H pylori</em></td>
<td>R</td>
</tr>
</tbody>
</table>

MTZ: metronidazole, AMX: amoxicillin, TET: tetracyclin, ERY: erythromycin, LEV: levofloxacin, NOR: norfloxacin, COT: cotrimoxazole,

Antimicrobial resistance pertaining to various gastroduodenal diseases:

Metronidazole- All 05 isolates were found resistant in patients with duodenal ulcer, gastric ulcer, chronic gastritis

Amoxicillin- 03 isolates were resistant in patients with duodenal ulcer and 02 isolates were sensitive in patients with gastric ulcer and chronic gastritis

Norfloxacin-03 isolates were resistant in patients with duodenal ulcer and 02 isolates were sensitive in patients with gastric ulcer and chronic gastritis.

Tetracycline, erythromycin, levofloxacin and cotrimoxazole - One isolate was resistant in duodenal ulcer patient and remaining 04 isolates were sensitive in patients with duodenal ulcer, gastric ulcer and chronic gastritis.
DISCUSSION

*H. pylori* is the commonest cause of chronic gastritis, gastric ulcer, duodenal ulcer, gastric carcinoma and MALT lymphoma. Hence it is important to identify this infection at an early stage and treat the condition at the earliest stage to avoid complications.22,33

A total of 200 patients with acid peptic disease were enrolled in the study. Among them 50% of patients were in the age group of less than 40 years and 20% were in 51-60 years age group. Out of 200 patients, 76% were males and 24% were females.

In another study too, it was observed that the acid peptic disease was more common in younger age group of 20-40 years (48%) and 74% were males and 26% were females across all the age groups,33 which is in agreement with our study. In our study the endoscopic examination of the study population revealed that 95% of the patients had chronic gastritis and 5% of the patients had peptic ulcer. Subbukesavaraja et al,34 in their study found that duodenal ulcer accounted for 36%, gastritis in 30% and gastric ulcer in 17%.

In our study out of 200 study subjects, 18.5% of samples were positive by Gram’s staining method, 29.5% were positive by modified Giemsa staining, 26.5% were positive by urease test, 2.5% were positive by culture, 48.5% were positive by PCR for detection of *16S rRNA*, 31% were positive by PCR for detection of *cagA* gene and 14.5% were positive by H&E staining. H&E staining is taken as the gold standard according to CDC and compared with all other methods and calculated sensitivity, specificity, positive predictive value and negative predictive values.
In the previously reported study done by Madhu Sharma et al, histopathology was used as the gold standard in diagnosing \textit{H pylori} infection as culture has marked limitations. Information with regards to histopathology of the gastric mucosa is an important advantage as \textit{H pylori} is found in the gastric mucus on the surface epithelium as reported by Gurjeet Kaur et al.  

Testing of biopsy specimen for urease is the best indirect method for the identification of \textit{H pylori}. The rapidity with which the test becomes positive is related to the number of \textit{H pylori} present in the specimen. In our study majority of the positive biopsy specimens showed a detectable color change within 30 minutes though the tubes were observed for 24 hours. Out of 200 samples studied by rapid urease test 53 (26.5\%) were positive. The overall positivity of rapid urease test correlates with reports by Sivaprakash et al\textsuperscript{38} (38.7\%), Subbukesavaraja et al\textsuperscript{34} 43.21\%. However it is known that in the rapid urease test false-negative results may occur because of irregular distribution of bacteria in the gastric mucosa or the use of antimicrobials, PPI’s or in patients with active or recent bleeding.\textsuperscript{39} On the other hand the contamination of biopsy with saliva can cause false positive results because bacteria from the oral flora can produce urease.\textsuperscript{4,14}  

Modified Gram’s staining by using dilute carbol fuschin as counterstain was done on 200 gastric biopsy samples. 37(18.5\%) out of 200 samples were positive for \textit{Helicobacter pylori} which is comparable to the study by Subbukesavaraja et al\textsuperscript{34} 22.2\%, while Anjana et al\textsuperscript{40} reported 72.3\% whereas in many studies positivity rate in direct Gram’s stained smear of biopsy samples varies from 40-90\% as reported by various workers.\textsuperscript{37,41} Low positivity may be due to organisms hidden in proteinaceous material. In the present study modified Giemsa staining of the crushed smear was
positive in 59 (29.50%) samples out of 200 which correlates with study by Coudron et al, 42 31% and Subbukesavaraja et al,34 27.1%. The modified Giemsa stain is inexpensive, easy to perform, rarely requires repeat stains and easily reproducible. The major disadvantage of this method is that there is little contrast between organisms and tissue.43

In the present study, *H pylori* was isolated in 5 out of 200 patients (2.5%) with sensitivity of 13.79%. Similar isolation rate has been reported by Gaval et al 44 2%, Sharma M et al35 4.2%, Kaore NM et al 45 8.7% with sensitivity of 8.69%. Higher culture positivity rate has been reported by various workers. 46,47 But studies from India have shown low rates of isolation.48

In contrast the Indian studies reported a sensitivity which ranged from 1.09% (as reported by Ayyagari et al 49) to 63% (as reported by Akbar et al 50). The sensitivity of our culture is in accordance with these results. The low rate of isolation may be because of the patchy distribution of the organism, low density of bacteria, the loss of viability of the organism in the specimen during transportation, fastidious nature of *H pylori*, inadequate mincing of the biopsy material, the presence of oropharyngeal flora, atmospheric environment and the use of antibiotics or PPI’S.51,52,53 These factors are difficult to control. All these factors together result in low sensitivity and low negative predictive value associated with culture of *H pylori*.45,54 While culture is laborious and time consuming. It has the advantage of permitting antimicrobial susceptibility testing and molecular studies to be performed on the isolates. Antimicrobial susceptibility is now increasingly essential with increasing reports of resistance among *H pylori* isolates to the various antimicrobials used for therapy.55
In our study the presence of *H pylori* in gastric biopsy was detected by Haematoxylin and Eosin staining (H&E) in 14.50%. Based on histology the *H pylori* prevalence was very low at 8% according to the report by Gurjeet Kaur et al.\textsuperscript{36} Histopathology allows evaluation of the status of the mucosa. Sensitivity and specificity could be influenced by factors such as expertise or trained personnel and the levels at which the sections are taken. CDC recommends that histopathology (H&E) should be taken as gold standard. The chances of finding *H pylori* in biopsy specimens become less when the changes of chronic atropic gastritis are set in the stomach. These changes lead to absence or decrease in the *H pylori* load in the stomach probably due to lack of nutrients for this organism.\textsuperscript{56}

We found that a PCR assay that detects DNA encoding the *H pylori* 16S rRNA gene in formalin- fixed, paraffin –embedded biopsy samples had a high degree of positivity and accuracy for demonstrating the presence of the bacteria. This is one of the few studies in which a PCR assay was evaluated for a large number of patients (200).

Not long ago culture was the basic method for a bacteriologist who wanted to diagnose *H pylori* infection. However it is a fragile, slow growing bacterium and despite 100% specificity, many clinical laboratories are attempting to implement molecular diagnostic tests. Thus *H pylori* diagnosis will have benefit from the development of PCR technologies as well as the improvement of other molecular tests.\textsuperscript{57} Bacterial DNA can be used as a marker for infections. The potential advantages of PCR include high specificity, quick results and the ability to type bacteria whether they were in a viable form or not but practical considerations and costs have limited its use.\textsuperscript{58}
In our study *H pylori* 16S rRNA gene detection by PCR was positive in 97 (48.50%) out of 200 gastric biopsy samples with sensitivity of 96.55% and specificity of 59.65%. Stella Smith et al.\(^{59}\) in their study have reported positivity of 52.38% for 16S rRNA with sensitivity of 100% and specificity of 68% using histopathology as the gold standard. This is in accordance with our study.

In a previous report by Park et al.\(^{60}\) on the genotyping of *H pylori* from biopsy and compared with culture both methods were approximately equal. Our findings indicate that this molecular assay is specific for *H pylori* DNA in gastric biopsy specimens. The *H pylori* 16S rRNA primers used were designed from those tested by Weiss et al.\(^{61}\) who demonstrated the specificity of their primers in detecting *H pylori* DNA in paraffin-embedded gastric tissue. They found that these primers did not cross react with many other common microorganisms such as *E coli*, various *Campylobacter* species and other *Helicobacter* species including *H cinnaedi*, *H fenelliae* and *H muselae*. We found that the PCR assay targeting the 16S rRNA gene in biopsy specimens proved sensitive in detecting *H pylori* infection. It constitutes a very useful diagnostic tool for treatment follow up. Sometimes the number of bacteria in the gastric mucosa is usually small and may go undetected by culture or by other diagnostic methods.

The presence of certain genotype like *cagA* in infecting strains has been shown to be associated with a more dense inflammatory gastritis and peptic ulcer disease.\(^{62}\) PCR for *cagA* genotype of *Helicobacter pylori* using DNA isolated from gastric biopsy specimens was approximately equal to genotyping using bacterial DNA from cultures. Inconsistent results were associated with low *H pylori* density in biopsies.\(^{60,63}\) In our study *cagA* by PCR was detected in 62(31%) out of 200 gastric
biopsy specimens where 85.48% were having chronic gastritis and 14.52% were having peptic ulcer with sensitivity of 89.66% and specificity of 78.95%. Out of 200 gastric biopsy samples *H pylori* were isolated in 05 cultures. All these 5 (100%) *H pylori* isolates were positive for *cagA* and their corresponding gastric biopsy samples were also positive for *cagA* by PCR. In another study *cagA* was positive in 19 (45.24%) out of 42 gastric biopsy specimens with sensitivity of 92.9% and specificity of 78.6% with histology as gold standard. In accordance with our study. In a previous report on the genotyping of *H pylori* from biopsy and compared with culture both methods were approximately equal. The prevalence of *cagA* positive strains varies from one geographic region to another. It has been reported that *cagA* gene is associated with geographical variations and the severity of disease outcome. A study by Yamaoka and colleagues reported variability in the structural organization of the 3’region of *cagA* and it is associated with the severity of the disease. CagA gene typing from this study shows the value of differentiating pathogenic and non-pathogenic strains of *H pylori*. CagA detection by PCR from *H pylori* isolates is 100% where 40% had chronic gastritis and 60% had peptic ulcer. The result of *cagA* detection by PCR from *H pylori* isolates and their corresponding biopsies were identical. Similar results were reported by Mishra et al, in their study all 54 *H pylori* isolates and their corresponding gastric biopsy specimens were subjected to *cagA* PCR. The result of *cagA* PCR from *H pylori* isolates and their corresponding biopsies were identical; all biopsy specimens from *cagA* positive strains were also positive for *cagA*. Other reports also commented that the *cagA* positive *H pylori* strains increase Interleukin-8 production and gastric inflammation. The *cagA* was found in about 60-70% of *H pylori* strains in the West and these strains were associated with duodenal
Discussion

ulcer and gastric cancer. Nimri LF et al,\textsuperscript{68} have amplified \textit{cagA} genotype in 29(26.4\%) out of 110 gastric biopsies. They have also reported that \textit{H pylori} genotype had no significant association with either age or gender of the patient. Smith et al,\textsuperscript{59} detected \textit{H pylori} in 35\% of the biopsy specimens in Nigeria. Our results are in agreement with other studies. In a study on adult populations from Calcutta (India), high prevalence of \textit{cagA} (80-90\%) independent of disease status (with and without peptic ulcer disease) has been reported.\textsuperscript{69} Biopsy specimens from 91.7\% duodenal ulcer patients harboured \textit{H pylori cagA} positive strains where as 73\% of the patients with chronic gastritis were found to be \textit{cagA} positive.\textsuperscript{70} These findings indicate that the PCR is as sensitive as culture for diagnosing \textit{H pylori} infection and that the presence of \textit{H pylori cagA} gene can be detected directly in biopsy specimens by PCR amplification. No false-positive or false negative results were found for biopsy specimens amplified by the \textit{cagA} primers suggesting the absence of inhibitory substances in gastric biopsy specimens.\textsuperscript{70} The \textit{cagA} protein is present in 60-80\% of \textit{H pylori} strains and it is found in close association with production of vacuolating cytotoxin.\textsuperscript{71} The frequency of \textit{cagA} in \textit{H pylori} strains isolated from peptic ulcer patients is greater than in those isolated from chronic gastritis patients.\textsuperscript{72} It has been observed that positive \textit{cagA} status of \textit{H pylori} infection is epidemiologically associated with the development of peptic ulcer and with an increased tendency to develop atropic gastritis, intestinal metaplasia and gastric cancer.\textsuperscript{73} It has been said that \textit{H pylori} may have different consequences in different populations because of variation in strain, host or environmental cofactors.\textsuperscript{74}

FDA approved regimens for patients with peptic ulcer disease include traditional triple therapy consisting of Metronidazole 250 mg, Bismuth subsalicylate
262 mg two tablets and Tetracyclin 500 mg all taken four times daily for 14 days. Quadruple therapy (in which triple therapy is combined with use of a PPI) has been shown to be more efficacious and associated with fewer side effects than has routine triple therapy.\textsuperscript{75} \textit{H pylori} infection is difficult to cure and requires combination therapies for eradication. \textit{H pylori} strains show in vitro antimicrobial resistance to one or more agents and this may be the cause of eradication failure.\textsuperscript{76,77} Non compliance of the patient and location of the bacterium which is beneath the gastric epithelium are involved in the treatment failure.\textsuperscript{2,76,77}

The mechanism of Metronidazole resistance may be due to mutation of the chromosomal \textit{rdxA} nitroreductase gene not the acquisition of new resistance genes (eg plasmids or transposons).\textsuperscript{78} MTZ is mutagenic. MTZ is used frequently against a variety of illnesses in India. The present high MTZ resistance in Indian \textit{H pylori} strains can be due to the frequent use of MTZ generally at doses that may induce and select for resident resistant mutant strains of \textit{H pylori} without eradicating them.\textsuperscript{79}

Antibiotic resistance varies widely by geographic location. \textit{H pylori} antimicrobial resistance in association with geographical disparity is due to the level of use of these antimicrobial agents in different communities.\textsuperscript{80} Antibiotics especially MTZ, Amoxicillin and Tetracyclin are widely used in our region to treat other infections. Antibiotic susceptibility testing of \textit{H pylori} is equally exacting as that of its isolation from clinical samples. Metronidazole is an important antimicrobial agent used in the treatment of \textit{H pylori} infection.\textsuperscript{81} Determination of antibiotic susceptibility particularly to Metronidazole is very essential.\textsuperscript{82} In our study antibiotic susceptibility testing was carried out by modified disc diffusion method. Although the NCCL’S has suggested the agar dilution method of antimicrobial susceptibility as the test of
Discussion

choice and reference method for testing of *H pylori* however it is a laborious and time consuming method.

Moreover in several studies it is documented that there is a good correlation between modified disk diffusion method and E (epsilometer) test methods and both are equally very good alternatives to agar dilution method.

Also Xia et al, Decross et al and Chaves et al have shown that disk diffusion method is a good alternative for determining antimicrobial susceptibility of *Helicobacter pylori* particularly to Metronidazole.

In our study a total of 05 isolates of *H pylori* subcultured were tested for antibiotic susceptibility using modified Kirby-Bauer disc diffusion method on Mueller-Hinton agar plate supplemented with 10% sheep blood. In other studies also Mueller Hinton agar supplemented with sheep blood was used. Pandya HB et al, have also used Mueller Hinton agar supplanted with 10% human blood for testing Kirby Bauer disk diffusion method. In our study all the strains 05 (100%) were resistant to Metronidazole. In another study *H pylori* resistance rate was 77.9% to Metronidazole. The resistance of *H pylori* to Metronidazole within India showed a range of 37.5% to 100%. A report from Eastern India has shown high Metronidazole resistance of 85% in *Helicobacter pylori* strains. This is in accordance with our study. Balamourougane et al, have reported high native resistance to Metronidazole in view of its extensive use in treating parasitic diseases in India. They have opined Omeprazole, Amoxicillin as currently being most suitable for Indian patients who are infected with *H pylori*. A considerable difference has been observed for primary Metronidazole resistance between the various regions which may be because of extensive use of Metronidazole which is an inexpensive antibiotic for the treatment of parasitic, genital and dental infections especially in developing countries like India.
In our study 03 (60%) of the strains were resistant to Amoxicillin. Frequent use of this drug for other infections like respiratory conditions in our area may contribute to resistance. Pandya HB et al.\textsuperscript{31} reported high resistance for Amoxicillin (72.5%) in their region. In our zone, we got emerging resistance with Norfloxacin where three isolates of \textit{H pylori} showed resistance. The reason for high resistance with Norfloxacin is due to its widespread usage in the treatment of urinary tract infections.\textsuperscript{90} One resistance was seen with other drugs like Tetracycline, Levofloxacin, Cotrimoxazole and Erythromycin in our study. Where as in earlier studies from Mumbai \textsuperscript{91} reported Tetracycline resistance in 27% of the \textit{H pylori} isolates and from Lucknow\textsuperscript{92} reported Tetracycline resistance in 10% of the strains. The results are in accordance with our study. In the present study three isolates of \textit{H pylori} showed multiple drug resistance. In another study approximately 45% of the \textit{H pylori} isolates showed multiple drug resistance of double, triple and quadruple types.\textsuperscript{8} \textit{H pylori} antimicrobial susceptibility pattern to commonly used antibiotics varies from region to region. Sensitivity pattern variation among \textit{H pylori} strains from different parts of India could be due to the possibility that these strains acquired resistance to antibiotics differently. Metronidazole needs to be replaced in the \textit{H pylori} treatment regimen. It is therefore important to continue antibiotic resistance monitoring to obtain accurate information on local rates especially that of high level resistance to Metronidazole.
CONCLUSION

*H. pylori* infection is strongly associated with peptic ulcer disease. Majority of *H. pylori* infected individuals remain asymptomatic. Infection with *H. pylori* is considered as an identified risk factor for peptic ulcer. Gastritis was the most common form of acid peptic disease. Acid peptic diseases commonly affect the age group of < 40 years with male preponderance.

We found that the PCR assay targeting the cagA gene in gastric biopsy samples is a valuable technique for detection of *H. pylori*. Its high sensitivity of 89.6% and specificity of 78.9% makes it a reference test for detection of *H. pylori*. The amplification of *H. pylori* cagA gene detected from biopsy samples matched closely with their detection in corresponding *H. pylori* isolates. Thus PCR has a potential value for cagA detection directly from biopsy samples allowing rapid determination of patients who are at high risk for peptic ulcer.

Culture is the best method without any false positive results but the false negativity is highest with it. Since urease test has got high sensitivity, it can be used for rapid diagnosis of dyspepsia. Culture has high specificity, which can be used as an adjunct.

Due to marked variation in the level of antimicrobial resistance amongst *Helicobacter pylori* strains between different countries, it is important to carry out susceptibility studies in each population. The use of antibiotics for other indications seems to be the major risk factor for the development of primary resistance. The results emphasize the need to perform resistance patterns at regional levels and recommends the rotation of antibiotics in *H. pylori* treatment in India. Currently
Conclusion

Metronidazole needs to be urgently replaced in the *H pylori* treatment regimen in India.

Limitations

The results of this study showed low culture positivity in existing controlled conditions. Further long term studies need to be conducted by using the modified culture methods. *CagA* gene detection by PCR which is the marker of pathogenicity was included in our study but other *H pylori* virulence associated genes like *vacA* and *iceA* need to be explored.

Recommendations

According to our antibiotic susceptibility testing, all the strains showed resistance to Metronidazole. Hence we suggest gastroenterologist not to include Metronidazole in the treatment regimen.
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