A study on phenotypic characterization and genotypic determination of drug resistance of *Helicobacter pylori* from cases of acid peptic disease in a tertiary care center

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INTRODUCTION

The identification of *Helicobacter pylori* and its significant role in gastric diseases originated from simple observations of spiral organisms in the stomach lining of humans and animals. Nowadays, *H. pylori* is a concern due to its direct association with gastroduodenal disorders. The prevalence of *H. pylori* exceeds 90% in developing countries, colonizing the stomachs of over half of the global population.¹ The prevalence rate varies depending on geographic location, socioeconomic status, personal hygiene, and age. Lifelong infection with *H. pylori* is a major risk factor for developing clinically significant diseases, including peptic ulcers, chronic gastritis, gastric carcinoma, and...
mucosal-associated lymphoid tissue (MALT) lymphoma. \(^2\)\(^3\) \(H. \) pylori can survive in the gastric mucosa for many years.\(^4\) This bacterium possesses classical virulence factors, such as outer membrane protein, urease, cytotoxin-associated gene A, and vacuolating cytotoxin A.\(^5\)

Several diagnostic tests are available to detect \(H. \) pylori infection, categorized as invasive and non-invasive techniques. However, the selection of tests depends on the laboratory’s resources and the clinical situation, whether for diagnostic or eradication purposes. Invasive tests require an upper gastrointestinal endoscopy and biopsy material for the examination, including rapid urease tests (RUTs), histopathological evaluation, culture, and polymerase chain reaction. The RUT detects the presence of \(H. \) pylori by measuring its abundant urease enzyme. Smear evaluation of specimens is another rapid diagnostic method. Culturing \(H. \) pylori is challenging, expensive, and requires specialized media due to its meticulous nature. Although culture is highly specific, it is less sensitive.\(^6\)

Polymerase chain reaction (PCR) is now widely employed to identify \(H. \) pylori in dental plaque samples, saliva, and tissue specimens. This method offers high specificity, quick results, and the ability to identify different strains of bacteria for pathogenic and epidemiologic studies. In addition, \(H. \) pylori has been increasingly developing drug resistance, particularly due to the widespread use of antibiotics such as Macrolides, quinolones, and Metronidazole. Endoscopic diagnostic procedures have been used less frequently. Alternative techniques include antibody detection methods and carbon-labeled urea breath tests (UBTs).\(^7\) Due to, their low cost, speed, and patient acceptability, serological tests are often employed for screening in epidemiological research. The most common and accurate non-invasive test for identifying \(H. \) pylori infection is the UBT. It can be used as a screening tool to evaluate eradication therapy’s efficacy and detect infection in children.\(^8\)^\(^9\)

The study’s goals were to determine the prevalence of \(H. \) pylori infection in dyspeptic patients, to assess the utility of the RUT, histopathology, and PCR for diagnosis, and to identify the prevalence of clarithromycin-resistant strains in the population by investigating genetic mutations through molecular analysis.

**Aims and objectives**

The aim of this study was to phenotypic identification of \(H. \) pylori and genotypic determination of drug-resistant \(H. \) pylori from suspected cases of acid peptic diseases in a tertiary care center.

**MATERIALS AND METHODS**

After receiving approval from the Human Institutional Ethical Committee, this 6-month prospective and observational study was carried out in the Department of Microbiology at Saveetha Medical College and Hospitals. Gastric biopsy samples were taken from 105 individuals with gastroduodenal disorders who had upper gastrointestinal endoscopy at the hospital.

**Inclusion criteria**

Specimens from inpatients and outpatients attending the surgery department and suggestive of upper gastrointestinal diseases, gastric ulcers, duodenal ulcers, antral gastritis, gastric carcinoma, and MALT lymphomas were included in the study.

**Exclusion criteria**

Patients with previous gastric surgery and active bleeding were excluded from the study.

Patients were allowed to provide informed consent before the endoscopy. They were told to fast overnight, and the endoscopy was done with a fiber optic endoscope. The endoscope and biopsy forceps were thoroughly rinsed with water before being immersed in 2% of glutaraldehyde for 20 min. The equipment was cleaned again with sterile normal saline before collecting the specimens. Patients’ throats were sprayed with lignocaine spray and positioned comfortably on their left side to reduce gagging. Before insertion into the mouth, the end of the endoscope was lubricated with lignocaine jelly. Patients were instructed to swallow until the scope reached the esophagus. The stomach and duodenum’s internal tissues were inspected for ulcers, erosion, inflammation, discoloration, and abnormal growth. Three biopsy samples were collected using biopsy forceps, preferably from the lesion site and the stomach antrum.

Four biopsy samples were taken from the antrum, 2 cm from the pylorus, and placed in sterile Eppendorf tubes. For rapid urease testing, one sample was inoculated into urea broth. Another specimen was collected in normal saline for gram staining, another in 70% ethanol for PCR, and the last in 10% formalin for histopathological examinations.

The biopsy sample was added to the broth, and the tubes were incubated at 37°C for rapid urease testing. Positive results were reported in the tissue sample that became pink within 20 min, indicating a rise in pH caused by \(H. \) pylori ammonia production. The slides were air-dried, heat-fixed for gram staining, and stained with methyl violet, Gram’s iodine, acetone, safranin, and water. After that, the slides

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were blotted dry and examined using an oil immersion objective. *H. pylori* was identified as Gram-negative curved bacilli. One specimen was fixed in 10% formalin for histopathological examinations, and paraffin sections were prepared and stained with Hematoxylin and Eosin (H&E) to examine *H. pylori*. *H. pylori* detection in gastric biopsies often involves various staining techniques to improve diagnostic accuracy. While routine H&E staining can reveal heavy bacterial loads, special stains such as Giemsa, Gimenez, Steiner, Warthin Starry, PAS-AB, Toluidine blue, and immune stains are valuable for enhancing the sensitivity and specificity of *H. pylori* detection. However, in our study, only H&E staining was done. The HELINI *H. pylori* real-time PCR Kit, a nucleic acid amplification real-time PCR kit, was used to detect and quantify *H. pylori* in human biological samples. Specificity, dynamic range testing, and PCR testing were performed.

**Detection of *H. pylori* using PCR**

PCR was done using a Purefast Bacterial DNA (Tissue) mini spin purification kit, and it detected *H. pylori* by identifying the urea gene. Clarithromycin resistance gene and point mutations were detected by agarose gel electrophoresis.

**Statistical analysis**

Data collected from patients were entered into an MS Excel sheet for analysis. Patients were categorized based on demographic details and clinical conditions. Quantitative data were represented using percentages, figures, and tables. SPSS 21.0 was used for conducting the statistical analysis and data visualization.

**RESULTS**

Of the 105 patients examined with upper gastrointestinal symptoms, 78 (74%) were male and 27 (26%) were female.

The plurality (40%) of the cases studied were between the ages of 51 and 60, with 25% older than 61. Endoscopic examination indicated gastritis symptoms in 70.48% of the patients, including antral, fundal, diffuse, or erosive gastritis. Gastric ulcers were discovered in 16.19% of patients, while duodenal ulcers were found in 1.9%. Based on stomach growth, two individuals were diagnosed with gastric cancer. The remaining 9.52% of patients had normal results or other problems, such as polyps (Table 1).

The age group 51–60 years had the highest proportion of PCR-positive *H. pylori* cases (48.28%), followed by the 31–40 year group (17.25%). Most instances (3.4%) were reported in people under 20 (Table 2 and Figure 1).

Twenty cases were positive by RUT, gram stain, and histopathology, and seven cases were positive by RUT and gram stain alone. Five cases were positive by RUT and histopathology alone. All cases of *RUT* were positive and showed either gram stain or histopathology positive or both positive (Table 3).

About 30.47% of the cases (n=105) were positive by RUT. Seventeen samples tested by RUT showed positive in patients with gastritis. Out of 17 patients with gastric ulcer, 10 turned rapid urease positive. Duodenal ulcer and carcinoma showed 2 and 1 RUT positive. About 25.1% of *H. pylori* infection was detected by Gram stain. Nine (52.94%) out of 17 patients with gastric ulcers were positive.

### Table 1: Demographic data of the study

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>78</td>
<td>74</td>
</tr>
<tr>
<td>Female</td>
<td>27</td>
<td>26</td>
</tr>
<tr>
<td>Age group (Years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11–20</td>
<td>2</td>
<td>1.9</td>
</tr>
<tr>
<td>21–30</td>
<td>9</td>
<td>8.5</td>
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<tr>
<td>31–40</td>
<td>15</td>
<td>14.3</td>
</tr>
<tr>
<td>41–50</td>
<td>10</td>
<td>9.5</td>
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<tr>
<td>51–60</td>
<td>42</td>
<td>40</td>
</tr>
<tr>
<td>&gt;61</td>
<td>27</td>
<td>25.7</td>
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<tr>
<td>Endoscopic diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastritis</td>
<td>74</td>
<td>70.48</td>
</tr>
<tr>
<td>Gastric ulcer</td>
<td>17</td>
<td>16.19</td>
</tr>
<tr>
<td>Duodenal ulcer</td>
<td>2</td>
<td>1.9</td>
</tr>
<tr>
<td>Carcinomatous growth</td>
<td>2</td>
<td>1.9</td>
</tr>
<tr>
<td>Others</td>
<td>10</td>
<td>9.52</td>
</tr>
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</table>

### Table 2: Categorization based on age for *H. pylori*-positive cases

<table>
<thead>
<tr>
<th>Age group in years</th>
<th>No. of cases</th>
<th><em>H. pylori</em>-positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;20</td>
<td>2</td>
<td>1 (3.4)</td>
</tr>
<tr>
<td>21–30</td>
<td>9</td>
<td>3 (10.34)</td>
</tr>
<tr>
<td>31–40</td>
<td>15</td>
<td>5 (17.24)</td>
</tr>
<tr>
<td>41–50</td>
<td>10</td>
<td>2 (6.9)</td>
</tr>
<tr>
<td>51–60</td>
<td>42</td>
<td>14 (48.28)</td>
</tr>
<tr>
<td>&gt;61</td>
<td>27</td>
<td>4 (13.8)</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>29 (100)</td>
</tr>
</tbody>
</table>

Figure 1: Age for *Helicobacter pylori*-positive cases
for Gram stain. Fifteen were positive in gastritis patients. Both patients with duodenal ulcers showed positive gram stains. One out of the two patients with carcinoma showed positive.

Histopathologically, 12 cases of *H. pylori* were detected in patients with gastritis and ten cases with stomach ulcers. Histopathology revealed positive results in both cases with duodenal ulcers. One out of every two cases of cancer tested positive. By PCR, 21.62% (16) of the 74 gastritis patients were positive for *H. pylori* infection. *H. pylori* was found in 52.94% and 50% of gastric and duodenal ulcer patients, respectively. *H. pylori* infection was found in one of the stomach cancer patients. *H. pylori* was found in two out of ten additional instances. There was a strong association between *H. pylori* infection and peptic ulcer disease compared to non-ulcer dyspepsia and CA stomach.

Rapid urease test and PCR gave comparable results for detecting *H. pylori*. Twenty-nine out of 32 RUT-positive samples were confirmed by PCR by detecting the specific *H. pylori* urea gene (Table 4 and Figure 1).

We discovered one new point mutation at T2243C. We found no point mutations at locations 2142 or 2143. Five of the 29 isolates tested positive for mutation at the T2243C site of the 23S rRNA. Line 1 shows negative control, line 2 shows ladder 100 bp DNA, and S1 to S6 denotes the samples. Sample 5 shows the band at 288 bp fragment (S5) (Figures 3-5).

**DISCUSSION**

The study included 105 individuals with upper gastrointestinal symptoms, 74% of whom were men and 26% of whom were women. This observation is consistent with the findings of Subbukesavaraja and Balan who discovered that 72.4% of biopsy specimens were from males and 27.6% from females. However, other research demonstrated that females have a higher prevalence of *H. pylori* infection. For instance, Khalifehgholi et al. reported female enrolment was higher than male enrolment. In our study, 72.41% of the 29 patients infected with *H. pylori* were male, whereas 27.89% were female, indicating a higher frequency of infection in male patients. This discovery agrees with the findings of Yoosuf et al., Archana et al., and Reddy et al. Most patients in this study were between the ages of 51 and 60. Although *H. pylori* infection is frequently associated with children, our study focused exclusively on adults. As a result, 75% of patients with gastrointestinal illnesses and *H. pylori* infection were between 30 and 70. A study by Reddy et al. had a similar distribution of cases. In our location, the prevalence of *H. pylori* in the gastric mucosa of dyspeptic patients was 27.61%, contrary to popular opinion in the Western world, where the prevalence is claimed to be 70–90% among Asians. Similar results were seen in studies by Chattopadhyay et al., (50%) from Kolkata and Archana et al., (48%) from Bagalkot. However, a higher prevalence of 58–70% was observed in a study by Arora et al. The prevalence of infection can differ in various factors, including clinical condition, region and type of microorganisms, resistance patterns, and antibiotic susceptibility. Based on the literature, we have seen a variation in the age group of individuals.

The endoscopic examination revealed that gastritis accounted for 70.4% of the cases, followed by gastric ulcer.

**Figure 2:** Urease and *Helicobacter Pylori*-positive in gram stain, histopathology, and polymerase chain reaction

**Table 3: Correlation with combination of tests**

<table>
<thead>
<tr>
<th>Rapid urease test</th>
<th>Gram staining</th>
<th>Histopathology</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>20</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table 4: Comparison of urease with *Helicobacter pylori* positivity in gram stain, histopathology, and PCR**

<table>
<thead>
<tr>
<th>Clinical status</th>
<th>No. of cases</th>
<th>Rapid urease test</th>
<th>Gram stain</th>
<th>Histopathology</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastritis</td>
<td>74</td>
<td>17</td>
<td>15</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Gastric ulcer</td>
<td>17</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Duodenal ulcer</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Carcinomatous growth</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Others</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>32</td>
<td>27</td>
<td>25</td>
<td>29</td>
</tr>
</tbody>
</table>

PCR: Polymerase chain reaction
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Of the 105 samples tested using the RUT, 30.4% were positive. Similar results were seen in the findings of Sivaprakash R et al., reported (38.7%). Gram-stained smears (direct) in our study demonstrated a positivity rate of 25.7%, similar to the study conducted by Arora et al., and Subbukesavaraja and Balan, who reported 20% positivity. However, Archana D et al., reported a higher positivity rate of 72.3%. PCR found *H. pylori* in all five histopathologically negative biopsy samples and four biopsy specimens that were Gram stain-negative but RUT positive in both cases. PCR found *H. pylori* in 20 samples, with positive results for RUT, Gram stain, and histopathology. According to Clayton et al., conventional tests reported negative reports in seven of the 23 PCR-positive samples. All histopathological and Gram stain-positive tissues tested positive for fast urease. The differences in patients can be attributed to antibiotics use and other indications, including respiratory tract infections and dual resistance patterns, which carry a high risk for first-line therapy failure.

Five of the 29 *H. pylori*-positive PCR cases had mutations in the 23S rRNA gene at position 2243, indicating a clarithromycin resistance rate of 17.24%. Fontana et al., reported similar findings, in which point mutations at positions 2142 and 2143 were not discovered, but a T2717C point mutation was reported. A study conducted by De Francesco et al., concluded that the antibiotic resistance rate of *H. pylori* was 17.2% for clarithromycin, which aligns with our research. However, the clarithromycin resistance rate varies globally, ranging from 49% in Spain to 1% in the Netherlands. While this might offer some insight into our observations, it also suggests that in cases where patients have mixed infections and receive antibiotic treatment, the naturally selected resistant strains could exacerbate the issue of drug resistance.

Interestingly, the T2243C mutation was found in all clarithromycin-resistant isolates in our region. While this study implies that T2243C plays a significant role in clarithromycin resistance, other mutations could be at work. In their investigation, Khademi et al., discovered similar mutations in the 23S rRNA gene at position T2243C.

**Limitations of study**

The study’s limitations include a small sample size (105 patients) and single-center design, potentially limiting generalizability. Lack of long-term follow-up and absence of a control group may hinder comprehensive...
insights into *H. pylori* infection’s impact on disease progression. Diagnostic methods used, though varied, might have inherent limitations affecting accuracy. To strengthen findings, future research should consider larger samples, multi-center studies, longer follow-ups, and the incorporation of updated diagnostic methods.

**CONCLUSION**

*H. pylori* was found in 27.67% of individuals with acid-peptic illness in this location. An in-house RUT was utilized to detect *H. pylori* infection, which is a cheap, simple, and quick method. Although culture is regarded as the gold standard, it is difficult, costly, necessitates an enriched transport medium, and yields delayed results. *H. pylori* PCR is a highly effective, high-detection approach. As a result, combining RUT and PCR is a valuable diagnostic strategy. Fluoroquinolone-containing second-line drugs, such as levofloxacin and moxifloxacin, are utilized as alternatives to clarithromycin in drug-resistant individuals.

In conclusion, we discovered a new point mutation, T2243C, in the *H. pylori* 23S rRNA gene that contributes to clarithromycin resistance in our isolates. This mutation pattern shows geographical variations. Further research incorporating the full 23S rRNA gene is required to uncover other possible point mutations.

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**REFERENCES**


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Authors Contribution:
MER – Manuscript preparation, performed the procedure; PS – Review Manuscript, RSAG – Protocol review, Review Manuscript; RDS – Literature review, Data collection, Data analysis; and NMR – Editing Manuscript.

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