ISOLATION OF HELICOBACTER PYLORI
FROM GASTRIC BIOPSY SPECIMENS

Vijayan K.T.V “A study on immunological and biochemical changes associated with helicobacter pylori infection” Thesis. Department of Biotechnology, University of Calicut, 2004
Chapter III

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Gastric pH of healthy humans is below 2 and this makes the colonization of microbes within the stomach difficult. Except for the presence of microbes originating from food, the human gastric content is sterile. This led to the thought that stomach is free from infectious diseases. But the successful isolation of H. pylori from gastric biopsies by Marshall and Warren (1984), and the accumulated report on its association with various gastroduodenal pathologies abolished this concept.

The key factor in the success of cultivation of H. pylori from gastric biopsy was the establishment of microaerophilic conditions originating from the technique applied for Campylobacters. Marshall and Warren (1984) inoculated the minced gastric tissue on non-selective blood agar and chocolate agar and incubated at 37°C under microaerophilic conditions. The colonies were developed on the plates after four days of incubation. These colonies were identified as H. pylori.

In tissue sections H. pylori appears as unipolar, multiflagellate, rod-like organism with bluntly rounded ends. They measure 0.5-1.0 μm in width and 2.5-4.0 μm in length (Goodwin et al., 1985; Jones et al., 1985). Direct observation on colonizing human gastric mucosa, it is a curved, sinuous or gently spiral bacterium (Goodwin and Armstrong, 1990). The sheathed flagella that terminate in a bulbous disc structure are the most distinguishing structural characteristic of the organism (Goodwin et al., 1985).

In cultures, H. pylori occur usually in the form of straight or slightly curved rods. Prolonged culture give rise to emergence of coccoid forms. Such forms occur in the faeces of the infected subjects. These are usually non-culturable forms of H. pylori (Bode et al., 1993). Organisms in the stationary phase of culture of H. pylori changed
their morphology from the spiral to coccoid form markedly under the anaerobic conditions (Shirai et al., 2000). Cellini et al. (1994) demonstrated the viability of the coccoid form by using BALB/c mice model. The conversion of coccoid form to spiral form and the recovery of urease activity and the ability to be subcultured on solid media were reported by Anderson et al. (1997). Thus, the coccoid forms of H. pylori were thought to be dormant form of the bacterium, which can revert to spiral form when conditions are appropriate.

The growth of H. pylori was improved by the incorporation of blood in the culture media (Caudron and Kirby, 1989). For the primary isolation of the organism from clinical specimens, blood based solid media were used. Marshall and Warren (1984) used chocolate agar for the isolation of the organism. Dunn et al. (1991), Doig et al. (1992), Lin et al. (1992), Pronovost et al. (1994), Cellini et al. (1994) and several others used chocolate agar for the cultivation of H. pylori. Skirrow's blood agar (Bolin et al., 1995; Nishiya et al., 1999). Blood agar (Kawanishi et al., 1995; Evans Jr. et al., 1989. Pan et al., 1997; Xiang et al., 2000). Brain heart infusion agar (Young et al., 2000; Rasko et al., 2000). Mueller-Hinton agar (Kostrzynska et al., 1991) were also used for primary isolation. Nakao et al. (1997) used Trypticase soy agar containing 5% sheep blood and Taylor (1988) used Brucella agar containing 10% sheep blood for culturing H. pylori. An indicator medium, Belo-Horizonte Medium (BHM), was developed (Queiroz et al., 1987) by incorporating triphenyl tetrazolium chloride to brain-heart infusion sheep blood agar. On this medium H. pylori developed colonies with unique golden pigmentation.

Some authors used blood free media for the cultivation of H. pylori. Morshed et al. (1994) obtained good growth of the organism in Brucella broth supplemented with 1% heated horse serum and 0.1% β-cyclodextrin. Egg yolk emulsion agar is another blood free medium reported (Westblom et al., 1991).
Testerman et al. (2001) reported chemically defined medium (F-12 agar), which supported the growth of *H. pylori*. F-12 agar plus cholesterol or β-cyclodextrin represents the first transparent chemically defined medium and the first urease indicator agar for *H. pylori*.

*H. pylori* grow slowly in broth media (Westblom et al., 1991). Contaminating microorganisms usually grow much faster than *H. pylori* and make liquid media useless for primary isolation of *H. pylori* from gastric biopsies.

In the absence of inflammation *Helicobacter* species appeared to be a part of a complex, indigenous microbial flora found in the gastric biopsy specimen (Monstein et al., 2000). Several authors used antibiotic supplement in the media for inhibiting the growth of contaminants. *H. pylori* are naturally resistant to vancomycin, cefsulodin, trimethoprim and sulfonamides (Goodwin and Armstrong, 1990). Commonly vancomycin, trimethoprim and nalidixic acid were used as antibiotic supplements in the media for the selective isolation of *H. pylori*. Resistance to nalidixic acid has been reported as a feature of *H. pylori*, but a few strains are sensitive. Taylor (1988) observed that most strains grown on 40 mg/L of nalidixic acid, but all are inhibited at 48 mg/L.

Primary cultures of *H. pylori* have less oxygen tolerance than most *Campylobacter* species, with a growth maximum at 3-7% of oxygen (Andersen and Wadstrom, 2001). Usually *H. pylori* were grown in jars with gas generating kits (Marshall and Warren, 1984; Anderson et al., 1997; Young et al., 2000) or in a CO₂ incubator with a standard microaerobic atmosphere (Kostrzynska et al., 1991; Testerman et al., 2001).

Most of the authors used gastric biopsies for the isolation of *H. pylori*. But there are reports showing the presence of *H. pylori* in oral cavity and faeces. Krajden et al. (1989) and Cellini et al. (1995) reported the isolation of *H. pylori* from dental plaques. They also reported that the isolates were identical with that colonized in the stomach of
respective patients. Ferguson et al. (1993) isolated \textit{H pylori} from saliva. \textit{H. pylori} were cultured from gastric juice of the patients (Young et al., 2000). Some authors isolated \textit{H. pylori} from faeces also (Thomas et al., 1992). But in all these cases the rate of isolation was very poor. Therefore, the major specimen that is used for the isolation of \textit{H. pylori} is gastric biopsies.

For inoculation, the biopsy sample can be rubbed several times on solid media (Jones et al., 1984), but mincing or grinding in the transport medium with a glass grinder results in heavier, more uniform growth (Goodwin et al., 1985).

**RESULTS**

Biopsy samples were collected from 41 patients with different gastroduodenal symptoms as per the endoscopy findings (Table 3.1). These patients were at the age range of 15-80. Of the 41 samples, majority was of duodenal ulcers. Two samples were from normal gastric mucosa.

<table>
<thead>
<tr>
<th>Endoscopic findings</th>
<th>No. of cases</th>
<th>RUT positive</th>
<th>Culture positive</th>
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<tbody>
<tr>
<td>Duodenal ulcer</td>
<td>13</td>
<td>08</td>
<td>04</td>
</tr>
<tr>
<td>Healing duodenal ulcer</td>
<td>16</td>
<td>07</td>
<td>04</td>
</tr>
<tr>
<td>Duodenitis</td>
<td>06</td>
<td>05</td>
<td>03</td>
</tr>
<tr>
<td>Gastritis</td>
<td>02</td>
<td>02</td>
<td>01</td>
</tr>
<tr>
<td>Mucosa oedematous</td>
<td>01</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Fundal carcinoma</td>
<td>01</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Normal</td>
<td>02</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Overall, 53.65% cases (22/41) showed positive reaction in rapid urease test (Table 3.2). The positivity of the sample was defined as follows: those biopsy
specimens, which gave positive result within 6, 12 and 24 h were recorded as strong positive, moderately positive and weakly positive, respectively. Both biopsies from patients with normal mucosa were negative for rapid urease test and both gastritis biopsies were positive. 83.33% of duodenitis cases (5/6), 61.53% of duodenal ulcer cases (8/13), and 43.75% healing duodenal ulcer cases (7/16) were positive for rapid urease test.

### Table 3.2 Rapid urease tests in Biopsies

<table>
<thead>
<tr>
<th></th>
<th>Total No. tested</th>
</tr>
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<tr>
<td>Strong positive</td>
<td>15</td>
</tr>
<tr>
<td>Moderately positive</td>
<td>05</td>
</tr>
<tr>
<td>Slightly positive</td>
<td>02</td>
</tr>
<tr>
<td>Negative</td>
<td>19</td>
</tr>
</tbody>
</table>

The colonies developed on the agar were analyzed and those colonies, which showed characteristic morphology and strong positive reactions for catalase, oxidase and urease tests were identified as *H. pylori* (Table 3.3). Further characterization of these colonies showed that they were unable to grow aerobically and does not utilized glucose or other carbohydrates. All isolates showed active cork-screw type motility.

Urease negative biopsies did not yield any *H. pylori* colonies. Total of 12 strains were isolated from rapid urease test positive biopsy samples.

Figure 3.1 shows the presence of *H. pylori* in the Gram-stained preparations of biopsy specimens used for the isolation of the organism. Typical morphology like slightly curved structure of *H. pylori* can be seen in the smear. Figures 3.2 and 3.3 shows the development of *H. pylori* colonies on chocolate agar and Gram-stained smear of isolated *H. pylori* colonies, respectively. Gram's stained smear from isolated
Figure 3.1 Gram stained preparation of biopsy showing *H. pylori*
Figure 3.2 Colonies of *H. pylori* on chocolate agar
Figure 3.3 Gram stained smear of colonies of *H. pylori*
colonies showed organisms similar to that of organisms present in biopsy samples. It may be noted that these colonies were isolated from the biopsies, which showed the presence of *H. pylori*.

Among the 12 isolates 4 were obtained from duodenal ulcer cases, 4 from healing duodenal ulcer, 3 from duodenitis and one from gastritis. Each isolates were stored in Brucella broth with foetal calf serum (20%) and glycerol (30%) at -20°C.

**Table 3.3** Gram's staining and biochemical characters of isolates identified as *H. pylori*.

<table>
<thead>
<tr>
<th>Character</th>
<th>Reaction</th>
</tr>
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<tbody>
<tr>
<td>Gram's staining</td>
<td>Gram-negative, slightly curved rods</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
</tr>
<tr>
<td>Urease test</td>
<td>Strongly Positive</td>
</tr>
<tr>
<td>Catalase test</td>
<td>Strongly Positive</td>
</tr>
<tr>
<td>Oxidase test</td>
<td>Strongly Positive</td>
</tr>
<tr>
<td>Ability to grow aerobically</td>
<td>No growth</td>
</tr>
<tr>
<td>Glucose utilization</td>
<td>Negative</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>Negative</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The majority of *H. pylori* organisms are free living in the mucus layer of the stomach overlaying the mucosal epithelium, a small proportion appear to adhere epithelial cells, and few, if any, actually invade tissue (Blaser and Parsonnet, 1994). *H. pylori* population is maintained at high concentration (about 10⁸ CFU/g) in gastric tissue for decades. But the distribution of the organism is not uniform and mostly patchy distribution was observed. So the sampling error could be leading to fallacious results.
According to Misra et al. (2000), two biopsies taken from A3 region of the antrum were sufficient for confirmation of the presence of *H. pylori* and associated gastritis. These biopsies should be taken from the most normal appearing mucosa (Graham and Qureshi, 2001).

Successful isolation of *H. pylori* from gastric biopsies relies on several factors, such as the transport medium, time in transport to the laboratory, temperature during transportation and medium used for isolation. Several media were recommended for transportation of the specimen. Normal saline or 20% glucose (Goodwin et al., 1985), Thioglycollate broth (von Wulffen et al., 1986; Queiroz et al., 1987), Brucella broth (Taylor, 1988), Carry-Blair medium (Kawanashi et al., 1995; Nakao et al., 1997), etc. were some of the common transport media used.

The temperature during transportation highly influences the viability of *H. pylori*. There was a significant decrease in viability of organism after 6 h at room temperature. At 4°C the organism survived for one week and at -70°C, or in liquid nitrogen, it will survive indefinitely (Graham and Qureshi, 2001). It was recommended to transport biopsy samples in transport medium at 4°C and processed within 4-5 h, if in glucose or saline (Rautelin and Kousunen, 1991).

*H. pylori* can be readily identified on the basis of characteristic colonial and microscopic morphology, a positive oxidase and catalase test, and the rapid hydrolysis of urea. Many authors solely depended on these characters to identify the organism in primary isolation (McNulty et al., 1989; Lin et al., 1992; Nishiya et al., 1999).

Different methods are used for the diagnosis of *H. pylori* infection. Histology provides marked evidence for *H. pylori* infection. *H. pylori* cells were present in sufficient quantities to be seen with high-power oil-immersion magnification of histologic sections or of Gram stains of smears of gastric mucus (Graham and Qureshi, 2001).
Based on the ability of the organism to produce large amount of urease, both non-invasive (urea breath tests) and invasive (rapid urease tests) tests were developed to diagnose *H. pylori* infection. Atherton and Spiller (1994) gave an overall view and importance of urea breath test for diagnosis of *H. pylori* infection. $^{13}$C-UBT gave reliable information about *H. pylori* status of the patient before or after therapy (Klein and Graham, 1993; Klein et al., 1996). Rapid urease test is widely accepted and it showed almost 100% specificity when compared with detection of *H. pylori* by Gram staining, culture and histology (McNulty et al., 1989).

HpSA is another non-invasive test developed and found to be easy-to-use test for diagnosing *H. pylori* infections (Vaira et al., 1999). Combined with UBT, HpSA can be used for confirmation of *H. pylori* eradication.

PCR techniques are valuable for diagnosing *H. pylori* infection. In a comparative study, van Doom et al. (2000) observed that PCR had highest diagnostic sensitivity followed by histology, culture and CLO tests. At the present time PCR remains a research tool for the diagnosis of *H. pylori* infection.

The isolation of the organism in the culture is 100% specific. The sensitivity of the culture varies from 50-99% depending on the laboratory and interest of the microbiologist (Graham and Qureshi, 2001).

The gastric ulcers were considered as an infectious disease and recommended therapy to switch from treating symptoms with antacids to antibiotic eradication of the infection (NIH consensus statement, 1994). But many treatment strategies failed to eradicate *H. pylori* from patients. This was mainly due to primary and acquired resistance of *H. pylori* strains to antimicrobial agents. Glupczynski and European multicentre study group on antibiotic susceptibility to *H. pylori* (1992) observed an overall resistance of metronidazole 26% in European countries, which ranged from 7% in Spain to 49% in Greece. Metronidazole resistance in France was 25%, but it increased rapidly reaching 50% in 1994 (Megraud et al., 1994). Abraham et al. (1997)
reported over ¾ of all strains isolated from Lucknow and Mumbai were metronidazole resistant. About 90% strains from Kolkata were metronidazole resistant (Mukhopadhyay et al., 2000).

The primary resistance to macrolides was much lower than that for metronidazole. Westblom and Unge (1992) observed less than 1% macrolide resistance. Wolle et al. (2002) reported 26.2% metronidazole resistance and 2.25% clarithromycin resistance in *H. pylori* strains isolated from North Eastern part of Germany.

Coexistence of metronidazole resistant and sensitive strains in patients with peptic ulcer and gastritis were reported (Loivukene et al., 2000). Among metronidazole resistant strains, combined resistance to clarithromycin, ciprofloxacin, and tetracycline was reported (Boyanova et al., 2000). van der Ende et al. (2001) showed the co-existence of clarithromycin resistant and sensitive strains with identical genotypes.

In this circumstance, it was suggested to test *H. pylori* susceptibility to antimicrobial agents and interpretation of the results before commencing antimicrobial therapy. Isolation of the strains from each patient is the only diagnostic procedure, which provides samples for antimicrobial susceptibility testing. More over no reports were available from Kerala showing the prevalence of *H. pylori* infection and successful isolation of the organism from patients. The above facts led to standardize the method of isolation of *H. pylori* from human gastric biopsy specimens and use them for further immunological studies.

The present investigation showed that about 54% of persons with different gastroduodenal symptoms were positive for *H. pylori* infection, as assessed by using rapid urease test (RUT), in and around Kozhikode District of Kerala State. Rapid urease test is one of the reliable invasive diagnostic tests for *H. pylori* infection. According to Lin et al. (1992) biopsy of the antrum at gastroscopy and rapid urease test were most useful for the initial diagnosis of *H. pylori* infection. The sensitivity and
specificity of different urease tests ranged from 95 and 100% respectively (El-Zimaity et al., 1995).

Rapid urease test requires a high bacterial density in the specimen. The photographs of the biopsy specimen show the presence of microorganism abundantly and because it is an ulcer specimen, it was speculated that these organisms are mainly *H. pylori*. The rapid urease test with these biopsies was strongly positive. Of the biopsies used for isolation of *H. pylori*, there were more samples from healing duodenal ulcer patients. Only 44% of these samples were positive for rapid urease test and could isolate only four *H. pylori* colonies. This could be explained as the patients were under anti-ulcer therapy and because the infection was brought under control by eradicating the organism from the site of infection.

In the present study the *H. pylori* colonies were developed only from those biopsy samples, which were positive for rapid urease test. Analysis of the morphological and biochemical characters along with standard strain obtained from Dr. A. Grob (Germany), confirms the identity of the isolates. Many authors applied colony characters, morphology and biochemical characters as criteria for identification of *H. pylori* (Hu et al., 1992; Yang et al., 1997; Loivukene et al., 2000).

Primary isolation of *H. pylori* from biopsy specimen is a difficult process. In specialized laboratories the isolation rates of 75-90% were achieved (Goodwin et al., 1985). This may be due to the fastidious nature of *H. pylori* and to a number of factors that are difficult to control, like patchy distribution of the organism on the gastric mucosa, contamination of biopsy forceps, presence of oropharyngeal flora, loss of viability of organism during transportation (Piccolomini et al., 1997).

Transport medium, time for transportation and temperature at transportation highly influences isolation of *H. pylori*. However, according to Morton and Bardhan (1995) transport medium used is irrelevant provided biopsy specimen can be rapidly transported for culture. They obtained 56% and 50% isolation rates when the specimen
cultured within 3 h after transportation in complex media or 0.9% saline respectively. A long transportation time decreases the number of the *H. pylori* especially after antibiotic therapy, and if the number of bacteria is low, culture may become false negative (Andersen and Wadstrom, 2001).

The major factors responsible for transformation of spiral shaped *H. pylori* into coccoid forms are nutrient deprivation, exposure to anti-ulcer drugs and antibiotics, extended incubation, pH, and attachment to the gastric epithelium (Andersen and Wadstrom, 2001). The coccoid forms are the non-culturable, senescent forms of *H. pylori*. Minimizing the subculturing can minimize the transformation of *H. pylori* into coccoid forms. The isolates were prepared in aliquots and stored at -20°C.

Culturing on solid medium is the standard technique used in most laboratories for the isolation of *H. pylori* from gastric biopsies. 12 isolates from 22 rapid urease test positive biopsies were isolated in the present study. The rate of isolation is about 55%. Gastric biopsies taken during endoscopy have been reported as the best samples for the isolation of the *H. pylori* from patients (Rautelin and Kousunen, 1991). The culture results obtained from gastric biopsies were highly dependent upon the accuracy of the biopsy sampling and the bacterial load in the tissue sample (Mobley et al., 1988).

Thus, from the morphological and biochemical characters, it was concluded that the isolates obtained from biopsy specimens were *H. pylori*. Approximately 50% of the ulcers are at least due to *H. pylori* infection.