Helicobacter pylori in Dyspepsia – Antibiotic Sensitivity and Virulence Patterns

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Abstract

Background : *Helicobacter pylori* is implicated in acute superficial gastritis, chronic atrophic gastritis, gastric carcinoma and MALT associated lymphoma. Though colonization can occur in normal individuals, treatment is given if the organism is associated with virulence factors like vacuolating toxin and cytopathic toxin as coded by *vacA* and *cagA* genes respectively. No causal relationship between non-ulcer dyspepsia (NUD) and *H pylori* has been established. This study was carried out to delineate colonizers from pathogens so that appropriate treatment can be planned.

Method : 100 patients were recruited, 62 with NUD and 38 age and sex matched controls. 4 gastric biopsies and a serum samples were taken from each patient. The biopsies were examined for *H pylori* by culture, histopathology, rapid urease test, PCR and serum for IgG, IgM and IgA.

Results : Culture showed 22.6% positivity and urease 19% among the test group. Histopathology showed 53.2% superficial gastritis and 30.6% chronic gastritis among the test group ($P < 0.001$), PCR for *H pylori* was positive in 48.3% and *vacA* and *cagA* were 29% and 22.6% respectively ($P = 0.001$) among the tests. IgG, IgM and IgA were 41.9%, 46.8% and 43.5% respectively.

Conclusion : The culture and rapid urease tests were highly specific with high positive predictive value but if negative, infection cannot be ruled out. Similarly IgA and IgM positivity has high positive predictive value for on-going infection where as IgG may be positive in old healed infections also. PCR assay in biopsy specimens is a valuable technique for detection of *H pylori* with high specificity and sensitivity. The presence of *vacA* and *cagA* genes can differentiate innocuous bystanders and potentially invasive organisms.

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Key Words: *Helicobacter pylori*, Culture, Dyspepsia, PCR, Serology

Introduction

Marshall and Warren first demonstrated a strong association between the presence of *Helicobacter pylori* and gastritis [1]. This organism is implicated in acute superficial gastritis, chronic atrophic gastritis, gastric carcinoma and MALT associated lymphoma [2]. In developing countries most infections occur in childhood with prevalence rate exceeding 50% at age 10 and 80% in adulthood [3]. Even though colonization with *H pylori* is common, the treatment is given provided organism is associated with virulence factors like vacuolating toxin and cytopathic toxin coded by *vacA* and *cagA* genes [4]. No causal relationship between NUD and *H pylori* was established even though organisms have been reported in 30-60% of the cases [5]. This study was carried out to delineate colonizers from pathogens so that treatment modalities for individual patients can be planned.

Material and Methods

62 patients with symptoms of dyspepsia were selected for the study. 38 age and sex matched controls were advised

endoscopy for other illnesses. A proforma was filled by the individual for detailed history, including symptoms and history of drug intake particularly NSAIDs, steroids and antibiotics. Those with positive history for the above drugs were excluded. After physical examination endoscopy was carried out. Four tissue bites were taken from the antrum and body of the stomach [6] and collected in (i) Cary-Blair medium (ii) 10% formalin (iii) Tris-EDTA buffer and (iv) 10% urea broth. Blood sample was collected for IgG, IgA and IgM antibodies to *H pylori*.

The tissue in Cary-Blair medium was homogenized and inoculated directly to a Bacto Campylobacter Agar kit Skirrow (Difco) and streaked for isolation [7]. It is a blood agar base II supplemented with vancomycin, polymyxin B, cephalothin, amphotericin B and trimethoprim to inhibit commensal bacteria and fungi [8]. The plates were incubated at 37° C in a Difco Gas Pak jar with disposable Campy pak for 10% CO₂, 5% O₂ and 85% N₂ to create microaerophilic conditions. Incubation was carried out for 3-5 days. The colonies of *H pylori* appear as non-haemolytic, flat, gray with irregular or raised or round edge and mucoid appearance. Colonies, with Gram-negative bacilli and darting motility in a wet mount were selected for further biochemical identification. Catalase, oxidase and urease

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positive colonies were identified as *H pylori*[9]. *Helicobacter pylori* ATCC 43504 strain was used as reference control. The strains were subjected to antibiotic sensitivity test by comparative disc diffusion method for clarithromycin, tetracycline, metronidazole and amoxicillin. The paraffin sections from the tissue in formalin were cut and stained with haematoxyline-eosin, Giemsa and toluidine blue. The sections were studied for type of mucosa, infiltrated with organisms, type and density of inflammatory cells, atrophic changes in the glands, metaplasia or malignancy [10]. The presence of curved, S-shaped bacteria in the lumen of mucus secreting glands and the surface of lining cells, resembling *H pylori* were considered positive. One gastric biopsy specimen was placed in 10% urea broth with phenol red pH indicator. The test was considered positive when solution changed colour to pink within 30 minutes indicating strong urease action considered positive for *H pylori*.

Enzyme immunoassay of *H pylori* IgG, IgA and IgM antibodies detection was carried out by ELISA using DIALAB(Germany) kits.

Multiplex PCR of gastric biopsy tissue

The tissue collected in TRIS buffer and the DNA was extracted by spin column methods with the solutions provided with the QIAGEN kit and processed for *16SrRNA*, *cagA* and *vacA* gene by multiplex PCR. The coding for *16SrRNA* was selected because the organisms were definitively distinguished from *Campylobacter pylori* and other bacterial genera and placed in a new genus on the basis of *16SrRNA* studies [7]. The primer for *16SrRNA* of *H pylori* was as follows: (5'-3' end)[11]. Primer A(Hp1) CTG GAG AGA CTA AGC CCT CC, Primer B(Hp2) ATT ACT GAC GCT GAT TGT GC. The Primers for *vacA* are as followsL (5'-3; end), Primer A(vcl)ATG GAA ATA CAA CAA ACA CAC, Primer B(vc2) CTG CTT GAA TGC GCC AAAC. The Primers for *cagA* are as follows: (5'-3' end), Primer A(cc1) GAT AAC AGG CAAGCT TTT GAG G, Primer B(cg2) CTG CAA AAG ATT GTT TGG CAG A. PCR was carried out by using PCR buffer, Taq DNA polymerase and dNTPs. The thermal cycle conditions were as follows: Initial Denaturation 3 min at 94°C, 3- step cycling: denaturation 0.5 – 1 min 94°C, annealing 0.5 – 1 min 56°C, extension 1 min 72°C(30 cycles), final extension: 10 min 72°C.

The PCR products were run on agarose gel electrophoresis. 1% agarose gel was prepared in Tris-Borate buffer containing 5µl of 0.01% ethidium bromide. Submarine electrophoresis was run at 50V for 2 hr. Positive and negative control were put along with 100 bp DNA ladder. The products of *16SrRNA*, *vacA* s2 phenotype and *cagA* are 109,286 and 349 base pairs respectively (Fig 1).

Results

A total of 100 patients in the age group 19-71 years were included in the study; 71 males and 29 females. The predominant complaints were pain in the epigastric region and dyspepsia. The patients having exclusive gastrointestinal symptoms were included in the test group and those unrelated illness included in the control group (Table 1). Only objective criteria for infection of *H pylori* was selected i.e at least 2 out

Fig. 1 : Multiplex PCR for *Helicobacter pylori*

Lane 1 : Negative control, Lane 2 : 100 bp DNA ladder, Lane 3 : Positive Control 16SrRNA: Lane 4 : *H pylori* 16SrRNA and *cagA*, Lane 5 *H pylori* without *cagA* and *vacA*: Lane 6 *H pylori* 16SrRNA with *vacA*

Table 1

Presenting complaints/diseases of test and control group

Diseases and complaints	Test groups (62)	Control group (38)
Pain in Epigastrium	59	
Dyspepsia*	53	
Hypothyroidism, Pott's spine	-	38
SLE, Nephropathy, CRF, Cirrhosis, etc		
Loose motion, Diarrhoea	25	
Loss of appetite, dysphasia	42	-
Duodenal ulcer, gastritis	42	-
Anemia, weight loss etc other	23	03

*Dyspeptic symptoms other than pain in epigastrium e.g. flatulence, belching, nausea, bloating, fullness of stomach, indigestion etc

of 5 tests must be positive to include the individual in the infected group [12].

Table 2 shows that test group had increased IgM (46.8%) followed by IgA and IgG. Increased superficial gastritis (53.2%) followed by chronic gastritis (30.6%) was found in the test group. Table 3 shows that culture showed maximum specificity 98.5% and urease 100% specific. IgM and IgA showed 95.5% and 94.4% specificity. PCR was both sensitive and specific at 84.8% and 90.5% respectively. *Helicobacter pylori* shows maximum sensitivity to tetracycline (71.5%) followed by amoxicillin (35.9%) (Table 4).

Discussion

H pylori infection in the stomach is an important etiological factor for chronic active gastritis and

Table 2**Comparison of various tests among test groups and controls**

Test parameter	Test group (n=62)	Control group (n=38)	P value
<i>H pylori</i> IgG antibodies	26 (41.9%)	8 (21%)	0.047
<i>H pylori</i> IgM antibodies	29 (46.8%)	3 (7.9%)	< 0.001
<i>H pylori</i> IgA antibodies	27 (43.5%)	4 (10.5%)	0.001
Urease	12 (19.3%)	0	0.004
Culture	14 (22.6%)	1 (2.6%)	0.015
Histopathological study			
Normal	9 (14.5%)	36 (94.7%)	< 0.001
Superficial gastritis	33 (53.2%)	1 (2.6%)	< 0.001
Chronic gastritis	19 (30.6%)	1 (2.6%)	0.002
Adenocarcinoma	1 (0.6%)	0	
<i>H pylori</i> in HPE	7 (11.3%)	0	0.081
PCR 16SrRNA	30 (48.3%)	7 (18.4%)	0.007
<i>vac A</i>	18 (29%)	0	0.001
<i>cag A</i>	14 (22.6%)	0	0.002
Both <i>cag A</i> and <i>vac A</i>	2 (3.2%)	0	

Table 3**Specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) of various tests in detection of invasive *H pylori* infection(%)**

	IgG	IgM	IgA	Urease	Culture	HPE	PCR
Specificity	89.3	95.5	94.4	100	98.5	55.8	90.5
Sensitivity	87.9	81.8	75.8	36.7	51.5	42.4	84.8
PPV	78.3	90.0	86.2	100	93.3	42.4	80.0
NPV	57.1	60.0	57.1	0	57.6	57.6	90.0

SG - Superficial gastritis; CG - Chronic gastritis

Table 4**Sensitivity of *Helicobacter pylori* to antibiotics (n=14)**

Tetracycline	10 (71.5%)
Amoxicillin	5 (35.9%)
Clarithromycin	4 (28.6%)
Metronidazole	1 (7%)

development of peptic ulcer disease [13]. Early detection of *H pylori* in gastric mucosa is critical in preventing more serious consequences [14]. The analysis of the endoscopic biopsy specimens carried out for the presence of *H pylori* by histological examination has low sensitivity and specificity [15]. At present two different methods are needed to determine whether a patient is infected with *H pylori* [12].

H pylori acquisition persists for years with tissue and serologic response to colonization. This organism is unlikely to be responsible for more than 10% of non-ulcer dyspepsia of stomach. However more than 90% of patients with duodenal ulceration carry *H pylori* which is significantly more than age matched controls [16]. 50 to 80% of patients with benign gastric ulcer are colonized with *H pylori*. The risk of gastric

cancer attributed to *H pylori* is about 60-80% [5]. In our study, the maximum patients in the test group had pain in the epigastrium, dyspepsia, loss of appetite, duodenal ulcer and gastritis. However in the control group, endoscopy was carried in cases of CRF, chronic hepatitis and hypothyroidism (Table 1).

Seropositivity for *H pylori* has been shown to correlate with the incidence of gastric cancer in studies carried out in many countries. Most of these methods provide satisfactory results [17]. ELISA is currently used for screening of patients affected by gastritis and peptic ulcer for acute active infection due to some *H pylori* virulent strains. Presence of IgA and IgM antibodies seem to be correlate with the acute phase of illness while IgG antibodies are present at different titres shortly after primary infection and last in the blood for many years after infection. The best antigen preparation must include *cagA* coded antigens, the vacuolating toxin, the heat shock proteins, the urease complex and certain adhesins[18]. In the patient groups, out of the 62 patients with positivity for *H pylori*, 41.9% were positive for IgG, 46.8% for IgM and 43.5% for IgA ($P = <0.05$ for IgG, < 0.001 for IgM and 0.001 for IgA). However out of 38 control patients IgG, IgM and IgA were present in 21%, 7.9% and 10.5% respectively. The positive predictive value and the negative predictive for IgG was 78.3% and 57.1%, for IgM it is 90% and 60% and for IgA it was 86.2% and 57.1% respectively. The control patients showing positive IgG were possibly infected earlier and may not be harbouring the organism at present, as these antibodies last longer in these patients. The positivity of IgA and IgM indicated acute and ongoing infection. The IgA and IgM positive people in the control group were negative for *H pylori* by other methods which could be due inadequate sampling.

In this study the rapid urease test was positive in 12% of all the patients but 19.3% in the test group. None of the controls were positive for the test indicating a sensitivity and specificity of 36.7% and 100% respectively. However in one Indian study the rapid urea test was positive in 24/25 biopsy specimens indicating 96% positivity. They had 84% positivity in gram stain of smear and 68% culture positivity but the number of specimen were small. Urease test may give false positivity after 24 hours due to other urease producing bacteria [20]. In this study urease positivity at one hour is taken to be more specific.

The bacterial culture is unquestionably the most specific but it is subjected to sampling errors and may give false negative results [21]. In this study 22.6% of the test group show culture positivity for *H pylori* with a sensitivity and specificity of 51.5% and 98.5% respectively for invasive infection (Table 2). Other

Indian workers had reported variable culture positivity i.e. 23.9%, 65.8% [22, 23]. The low rate of isolation is possibly due to sub-optimal quality of locally available media, administration of antibiotics, high contamination rate due to warm humid atmosphere, delay in processing and difficulty in achieving the required gaseous environment, which is critical for isolation. However advantage of culture is characterization and antibiotic sensitivity of isolates. The failure of culture to detect the bacterium in the biopsy samples which were positive by PCR assay could be due to loss of viability in transport or PCR may detect a controversial resting form of the bacterium which is not able to grow [24].

The antibiotic sensitivity testing in our study showed that 71.5% of the organisms were sensitive to tetracycline, 35.9% to amoxicillin, 28.6% to clarithromycin and 7% to metronidazole. In another Indian study, the workers found 95.8% were sensitive to tetracycline, 87.5% to erythromycin, 83.3% to metronidazole, 75% to amoxicillin and only 12.5% to ampicillin [20]. Treatment of *H pylori* – associated peptic ulcer disease with triple therapy always heals the mucosal lesion but fails to eradicate the microbe in about 10% of all cases. Antibicrobial resistance may in part account for treatment failures. Clarithromycin, an intracellular active antibiotic is one of the cornerstones in present ulcer therapy. More than 90% macrolide resistance is due to either of two transition mutations (A-G) at adjacent position 2143 and 2144 in one or both of the 23S rRNA genes of the bacterium [25].

PCR for 16SrRNA from the gastric biopsy tissues is difficult as the homogenisation of the tissue and digestion by proteinase K and incubation at correct temperatures are critical factors. PCR assay can detect the target DNA whether the bacteria is viable or not, if the gross breakdown of nucleic acid has not occurred. We got only 48.3% positivity of PCR from test group though 18.4% were positive in the control group ($P=0.007$), indicating quiescent infections. However in the multiplex PCR, the *vacA* and *cagA* were positive in 29% and 22.6% respectively, ($P=0.001$ for *vacA* and 0.002 for *cagA*) which were specific for pathogenecity indicators as none of them were positive in the control group. One of the workers used a rapid two-step PCR to specifically amplify 298-bp sequence [6]. The less number of organisms in the biopsy tissue or presence of *in vivo* *Taq DNA Polymerase* inhibitors can lead to false negativity of PCR.

In this study 53.2% were having a histological picture of superficial gastritis and 30.6% chronic gastritis (Table-5). The superficial gastritis was significant ($P<0.001$) as well as chronic gastritis $P=0.001$. The normal picture in 14.5% could be due to erroneous selection of biopsy

area. In 11.3% of the test group *H pylori* could be demonstrated microscopically with blue stain. The organism lie along the luminal surface of epithelial cells and they do not invade the mucosa. Distribution can be patchy or irregular. Even in heavily colonized stomachs, the organisms were absent from areas of intestinal metaplasia [10].

To conclude, histopathological examination of gastric antral and corpus biopsy with toluidine blue stain for *H pylori* associated with serological examination for IgA and IgM gives reasonable evidence of the infection in NUD cases. PCR with 16SrRNA, *cagA* and *vacA* primers will help in differentiating commensal from pathogens. Cultures will help in antibiotic sensitivity of the isolates.

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