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RESEARCH PAPER

Detection of *Helicobacter pylori* and its Antimicrobial Susceptibility Pattern from Gastric Biopsy Specimens

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Abstract

Background: Early detection of *Helicobacter pylori* (*H. pylori)* infection is essential for its treatment. Resistance to amoxicillin, clarithromycin and metronidazole has been on the increase in many countries. Phenotypic resistance is correlated with treatment failure. So, there is an urgent need to explore sensitivity of other antibiotics, such as levofloxacin to combat *H. pylori* infection.

Objective: The study was aimed to detect *H. pylori* from gastric biopsy samples and its susceptibility profile to commonly used antimicrobial drugs.

Methods: Gastroduodenal biopsy specimens were collected from 143 adult dyspeptic patients during March 2018-February, 2019, who attended the outpatient department of gastroenterology, Bangabandhu Sheikh Mujib Medical University (BSMMU) and Dhaka Medical College Hospital (DMCH), for endoscopy. *H. pylori* was identified by rapid urease test (RUT), *ureC* gene PCR, histological staining (Giemsa) and culture. From culture isolates antimicrobial susceptibility of clarithromycin, levofloxacin, amoxicillin and metronidazole were detected by disk diffusion method.

Results: The highest rate of *H. pylori* infection was found in the age group between 41-50 years (25.5%). According to case definition, *H. pylori* positive cases were 47 (32.9%) and *H. pylori* negative cases were 96 (67.1%). Thirty five *H. pylori* positive samples were subjected to culture and only 10 (28.6%) were positive. Among 10 culture positive *H. pylori* isolates, clarithromycin exhibited 20% resistance, levofloxacin 30%, metronidazole 30% and no resistance found to amoxicillin.

Conclusion: PCR based assays can be an alternative rapid approach for the detection of *H. pylori.* In this study, levofloxacin showed high resistance, a further larger study is required to confirm this finding.

Keywords: Rapid urease test, *ureC* gene PCR, Clarithromycin, Levofloxacin

Introduction

Helicobacter pylori colonised the stomach of over half of the world's population but only 10-20% of infected persons become symptomatic.[1] The most common symptom of *H. pylori* infection is dyspepsia and prevalence of *H. pylori* infection in adult dyspeptic patient in India was 32.9%.[2, 3] Prevalence of *H. pylori* infection was found to be high (60.2%) in Bangladesh, which is related to overcrowding and poor sanitary conditions.[4] *H. pylori* infection causes gastritis, duodenal ulcer, gastric carcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma.[5] Detection and treatment of *H. pylori* infection is recommended in all symptomatic individuals in order to prevent *H. pylori* induced diseases.[6]

Several invasive and noninvasive techniques are currently used for detecting *H. pylori* infection, such as rapid urease test (RUT), urea breath test, culture, PCR, serological tests and histopathology with their specific advantage and disadvantage.[7] Gastric biopsy specimens obtained during endoscopy are best sample for *H. pylori* isolation.[8]

Rapid urease test is the most useful invasive test for the diagnosis of *H. pylori* infection, sensitivity and

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specificity of various RUT tests as primary diagnostic tests vary between approximately 80 -100% and 97 - 99%.[9,10] Other than detection of *H. pylori,* histopathology also allows assessment of morphological changes of the gastric mucosa due to *H. pylori* infection.[1] Sensitivity and specificity of histology varies from 53% to 90%, depending on the clinical setting, density of colonisation and experience of the histopathologist.[11]

Many polymerase chain reaction (PCR) methods provides excellent sensitivity and specificity, as compared with other conventional tests for *H. pylori* detection from clinical specimens.[12, 13] *ureC* gene is more appropriate than other genes for detection of *H. pylori* from clinical specimens. The *ureC* gene later renamed as *glmM* gene, a housekeeping gene that encode the phosphoglucosamine mutase (*glmM*), which is unrelated to urease production.[12]

Although culture of *H. pylori* from gastric biopsy is the gold standard for antibiotic susceptibility testing, sensitivity of culture were lower as 55–73% in some studies, due to strain fastidiousness, effects of treatment, overgrowth of contaminating microorganisms etc.[14, 15] In routine practice, the detection of antibiotic resistance is mainly based on phenotypic methods: disk diffusion method, E-test or the agar dilution method.[14]

A triple therapy containing a proton pump inhibitor and two antibiotics, amoxicillin (AMX) and clarithromycin (CAM) or metronidazole (MNZ), is the standard first-line treatment of *H. pylori* infection in populations with less than 15–20% clarithromycin resistance.[6] In Bangladesh, clarithromycin based regimen is the first line *H. pylori* eradication therapy.[16] After failure of first line therapy, levofloxacin containing triple therapy is recommended as second line treatment.[17]

Clarithromycin, metronidazole and amoxicillin resistance rates had been reported to be 58.8%, 83.3%, 72.5% and 36.0%, 89.0%, 37% in India and Pakistan respectively.[18, 19] High resistance rate of *H. pylori* to levofloxacin (73.2%) has been reported in north India.[20] In Bangladesh 39.3% clarithromycin, 94.6% metronidazole, 3.6% amoxicillin and, 66.1% levofloxacin resistance rate had been reported in gastritis patients.[21]

As a recommendation from Maastricht IV Consensus Report, *H. pylori* culture and antibiotic susceptibility testing should be performed if primary resistance to clarithromycin is higher than 20% in a given geographical area or after the failure of second-line treatment.[22]

The present study was aimed to detect *H. pylori* by different invasive methods (histology, RUT, culture, and PCR) from gastric biopsy specimens, compare the sensitivity and the specificities of different *H. pylori* detection methods and, evaluate the resistance pattern to - clarithromycin, amoxicillin, metronidazole and levofloxacin in *H. pylori* by disk diffusion method.

Materials and Methods

This cross sectional, observational study was conducted over a period of 12 months starting from March 2018, at the department of microbiology & immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh. The study was ethically approved by the Bangladesh Medical Research Council, Dhaka.

Samples were collected from 143 adult patients, who presented with dyspepsia for more than 1 month, in outpatient department of gastroenterology, BSMMU and Dhaka Medical College Hospital (DMCH).[23] Patients above 65 years of age having severe medical or surgical illness, or history of intake of proton pump inhibitors, non-steroidal anti-inflammatory drugs, colloidal bismuth compounds, or antibiotics for eradication of *H. pylori* over the past four weeks of enrolment were excluded from the study.[24]

After obtaining written informed consent, a total of four pieces of gastric tissue were collected from lesion and surrounding sites of gastric antrum and body. First specimen was inoculated immediately into a screw capped bottle containing RUT media, change in the colur from yellow to pink within twenty four hours after incubation at 37ºC indicate positive test. Second biopsy specimen was collected in Stuart transport media for culture. Third biopsy specimen was collected in PBS for PCR and stored at -20°C until DNA extraction. Fourth specimen was fixed in 10% buffered formalin and transported to the department of pathology, BSMMU for histological examination.

A patient was considered as *H. pylori* positive case, if he/ she had, (i) Positive *H. pylori* culture[25] and / or (ii) At least two of the other three tests were positive.[26]

Culture and sensitivity testing: Gastric tissue was minced manually and placed on brain heart infusion

agar (Oxoid, Ltd, Basingstoke, Hampshire, United Kingdom) supplemented with 10% sheep blood, Vitamino Growth Supplement (Twin Pack) (Hi Media, India), and Campylobacter selective supplement (Hi Media, India).[27] The plates were incubated at 37ºC in an anaerobic jar with a gas generation pack (CO_2 Gen Sachet™, Oxoid, USA), and examined on 3rd day. *H. pylori* colonies were identified based on morphology (small, round, convex, translucent colonies) (figure1), Gram staining (curved rod), positive catalase, urease and oxidase test. If no growth observed on 3rd day, the plates were rechecked on every alternative day up to 7 days.

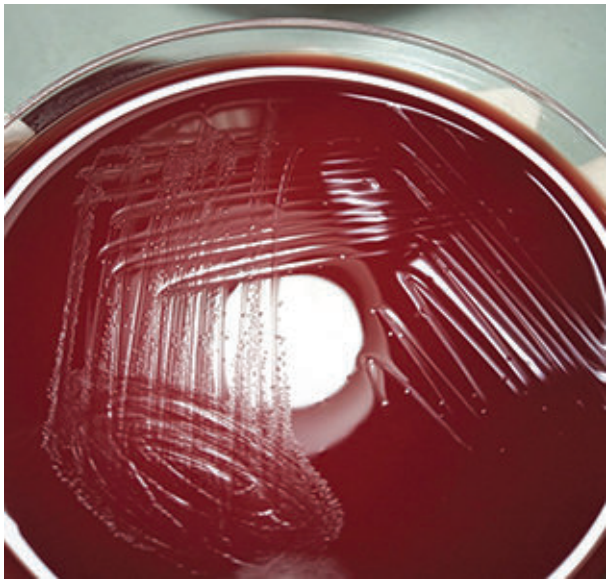

Figure 1: Growth of *H. pylori* in Brain heart infusion agar supplemented with 10% sheep blood, vitamino growth supplement and campylobacter selective supplement.

For antibiotic sensitivity testing, bacterial suspension was prepared and compared to McFarland 3 turbidity standard and inoculated in Mueller-Hinton agar plate supplemented with 10% sheep blood. After placing antibiotic disks, agar plates were kept at 37ºC for 72 hours under microaerophilic condition. Zone Diameter of clarithromycin (15 µg/disk) ≤18 milimeter (mm) [28], levofloxacin (5 µg/disk) <17mm [29], metronidazole (5 µg/disk) <16 mm[28], and amoxicillin (10 µg/disk) ≤18mm [30] were considered as resistant.

DNA was extracted from gastric tissues using QIAmp DNA mini kits (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The *ureC* primer sequences were - *ureC*-F 52 -AAGCTTTTA GGGGTGTTAGGGGTTT-32 , *ureC*-R 52 -AAGCTTACTTTCTAACACTAACGC-32 . The DNA were denatured at 94ºC for 5 minutes, followed by 35 cycles at 93ºC for 1 minute, 55ºC for 30 seconds, 72ºC for 1 minute and final extension at 72ºC for 10 minutes. Electrophoresis of the amplified products were done in 2% agarose gel at 110V for 42 minutes, amplified product were 294 base pair (figure 2).[12]

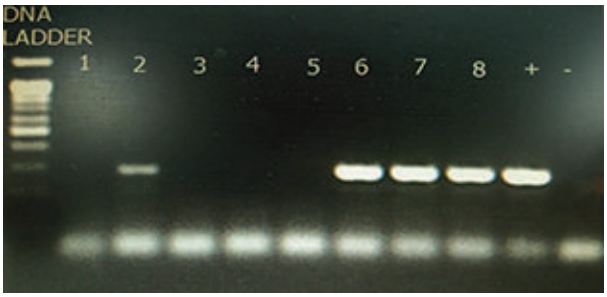

Figure 2: Agarose gel electrophoresis analysis showed amplified DNA product of *ureC* gene PCR (294 base pair). Lane 2, 6, 7, 8: positive. Lane 1, 3, 4 and 5: negative. Positive control: *ureC* gene positive *H. pylori*, Negative control: Amplified product of PCR without DNA

Data were re-checked and analysed using SPSS software version-23 (IBM, Armonk, New York). Sensitivity and specificity of different detection methods were calculated considering case definition of *H. pylori* positive cases as gold standard.

Results

A total of 143 gastric biopsy specimens were collected, rapid urease test, PCR amplification of *ureC* gene and histopathological examination were performed in all cases. Cultures for *H. pylori* were done only in 104 cases, as rest of the samples were not properly collected and transported for culture.

Among 143 cases, *H. pylori* was detected in 66 (46.2%) and 36 (25.2%) cases respectively by RUT and histological staining. *ureC* gene was detected in 42 (29.4%) cases, however cultures were positive only in 10 (9.6%) cases. According to the case definition of *H. pylori* positive case, out of 143 patients, 47 (32.9%) were considered as *H. pylori* positive cases. Among 47 *H. pylori* positive cases, 43 (91.5%) were found to be positive by RUT followed by *ureC* gene PCR 42 (89.3%) and histological staining 35 (74.4%). Whereas, 35 *H. pylori* positive samples were subjected to culture and only 10 (28.6%) of them yield culture positive (table I).

Table I: Rate of positivity of *H. pylori* by combination of rapid urease test (RUT), *ureC* gene PCR, culture and histological staining as per case definition (n=47)

Name of tests	Positive cases	Percentage
RUT, PCR, histology and culture	8	17.0
RUT, PCR and histology	18	38.3
RUT, PCR and culture	2	4.3
RUT and PCR	10	21.3
RUT and histology	5	10.6
PCR and histology	4	8.5
Total	47	100.0

Sensitivity and specificity of rapid urease test, histology, *ureC* gene PCR and culture for detection of *H. pylori* infection were calculated, considering case definition of *H. pylori* positive cases as gold standard (table II).

H. pylori was detected by culture only in 10 cases. Metronidazole and levofloxacin exhibited 30.0% resistance, whereas, clarithromycin resistance was 20.0%. All isolated *H. pylori* were sensitive to amoxicillin (table III).

Out of total 143 dyspeptic patients, 71(49.7%) were female and 72(50.3%) were male. Among the study population, male female ratio (1.00: 1.01) was almost equal. Among *H. pylori* positive cases 22(46.8%) were male and 25(53.2%) were female, so the male female ratio in *H. pylori* positive cases was 1.00: 1.14.

The age of the patients range from 18-65 years, with the mean age 42.3±14.3 years. The highest rate of *H. pylori* infection was found in the age group from 41-50 years (25.5%), while the lowest percentage of *H. pylori* infection (4.3%) was in the age group <20 years.

Histopathological examination of gastric mucosa revealed the highest number, 101(70.6%) patients had chronic gastritis among them 31(30.7%) were *H. pylori* positive. Whereas, only nine (6.3%) had duodenal ulcers amid five (55.5%) were *H. Pylori* positive. Among four intestinal metaplasia, 19 gastric ulcers, three adenocarcinomas, and seven normal gastric mucosa cases 50%, 39.8%, 33.3%, and 14.3% were *H. pylori* positive respectively.

Table II: Sensitivity and specificity of RUT, histology, *ureC* gene PCR and culture for detection of *H. pylori* infection

Detection methods	Sensitivity%	Specificity%	PPV%	NPV%	Accuracy%
RUT	91.5	77.0	65.1	95.1	83.9
Histology	74.5	98.9	97.2	88.8	90.1
ureC gene PCR	89.4	100	100	95.0	96.5
Culture*	28.5	100	100	73.4	75.9

*Cultures for *H. pylori* could not be done in 39 cases as the samples were not properly collected and transported for culture.

Table III: Antimicrobial susceptibility pattern of *H. pylori* isolates by disk diffusion method (n=10)

Antimicrobial agents	Sensitiven (%)	Resistantn (%)
Clarithromycin	8 (80.0)	2 (20.0)
Levofloxacin	7 (70.0)	3 (30.0)
Metronidazole	7 (70.0)	3 (30.0)
Amoxicillin	10 (100.0)	0 (0)

Discussion

Among 143 dyspeptic patients, 47 (32.9%) case were classified as *H. pylori* positive cases according to case definition of this study. This finding correlate with Niknam *et al* who reported 31% *H. pylori* positivity among adult dyspeptic population in Iran.[31]Aftab *et al* reported 47% *H. pylori* infection in adult dyspeptic patients in Bangladesh.[32] Several studies from Asian and Middle East countries reported declining *H. pylori* infection with improvement of hygienic condition.[33, 34] In present study, highest rate of *H. pylori* infection was found in the age group from 41-50 years (25.5%), similar to Helaly *et al*, who also found highest percentage of *H. pylori* infection in the age group from 41-50 years.[35]

Considering the case definition of *H. pylori* positive case as gold standard, *ureC* gene PCR showed 89.4% sensitivity and 100% specificity. Similar finding was reported by Khalifehgholi *et al* who reported sensitivity and specificity of *ureC* gene PCR 93.5% and 95.6% respectively.[36] Low density of *H. pylori* (fewer than 50 bacteria) on biopsy sample, presence of PCR inhibitory substance such as Taq polymerase, prolonged transportation or storage of sample at room temperature may produce misleading results with direct PCR from gastric mucosa.[25, 37] In this study, RUT and histology had 91.5% and 74.5% sensitivity and 77.0% and 98.9% specificity respectively. Closely similar finding were reported by Khalifehgholi *et al* who had reported 95.6%, 93.5% sensitivity and 100%, 77.8% specificity for RUT and Histology respectively in Iran.[36] Sensitivity of those tests varies with the number of biopsies obtained, localization of specimen, expertise of the pathologists, use of PPI and antibiotics.[8, 38]

In present study, culture showed 100% specificity but low sensitivity (28.5%). Wani *et al* reported sensitivity and specificity of culture to be 13.7% and 100% respectively for detection of *H. pylori* which coincide with this study.[39] Sensitivity of culture where higher 92.1% and 100% specific in another study of Aftab *et al.* [32] Fluctuation in sensitivity of culture may be explained by the absence or lower density of bacterium in the biopsy specimens, use of antimicrobials, fastidiousness of *H. pylori* or inappropriate conditions of transport.[8,40]

In this study, only 30% metronidazole resistance was observed by disk diffusion method. However, the higher incidence of 50%-80% metronidazole-resistant strains observed in other studies of developing countries.[41, 42]

In the present study, all isolated *H. pylori* were sensitive to amoxicillin. Similar finding was reported by Miftahussurur *et al*, who reported 100% amoxicillin sensitivity.[43]

Aftab *et al* and Islam *et al*, were also reported 3.6% and 7.3% amoxicillin resistance respectively in Bangladesh.[21, 23]

Clarithromycin resistance rate was 20%, this result was consistence with Gehlot *et al* and Sun *et al* in which resistance to clarithromycin were 11.8% and 20.7% respectively.[44,45] Aftab *et al* reported higher resistance rate of clarithromycin (39.3%) detected by agar dilution method.[21]

In present study, 30% levofloxacin resistance was observed, which coincide with the study of Miftahussurur *et al* who reported 42.9 % levofloxacin resistance. High prevalence (66.1%) of levofloxacin resistance was reported by Aftab *et al*. [43, 21]

Conclusion

Findings of this study demonstrated that, most sensitive methods for the detection of *H. pylori* infection were rapid urease test and *ureC* gene PCR. Clarithromycin and levofloxacin resistance rate were high in Bangladesh. Although, MIC of clarithromycin and levofloxacin by agar dilution method was not be measured as per CLSI guideline, as it is costly and difficult to perform. However, future multicentre studies on a large scale with a full characterisation of *H pylori* isolates are required to detect the prevalence of *H. pylori* resistance to commonly prescribed antimicrobials to confirm our findings and construct proper strategies to control or eradicate *H. pylori* infection.

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