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Helicobacter pylori cagA Genotyping by Restriction Fragment Length Polymorphism Isolated from Patients with Gastro-Duodenal Symptoms

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ABSTRACT

Helicobacter pylori colonize the gastric mucosa, related with different gastro-duodenal diseases and the clinical outcome linked to these diseases has been associated with pathogen virulence genes and their polymorphism. The aim of the study is to detect *Helicobacter pyloricagA* gene and to investigate the distribution of their genotypes in the patients with gastroduodenal symptoms. Total 51 patients were enrolled in the study on the basis of clinical and endoscopic findings. Written informed consent was obtained from each patient prior to the endoscopic procedure and collection of gastric biopsy specimens. Histopathological examination was done for detection of H. pylori in tissue specimens. The *H. pylori* histopathology positive specimens are considered as *Helicobacter pylori* positive and PCR were done for *cagA* detection and the positive specimens further tested by RFLP to detect the *cagA* polymorphism. Among the histopathology positive *H. pylori* cases 90% were positive for *cagA* gene by PCR and almost 100% *cagA* were β genotype by PCR-RFLP. No α genotype of *cagA* was found. Mean age of the patients were 46.9 ± 14.2 years starting from 22to 76 years. Out of 51 patients 39(76.47%) were male and 23.52% were female. According to the age group distribution, 22 (43.13%) were in 41-60 years age group and 39.21%and 17.64% were in 20-40 years and 61-80 years age group respectively. Among the 09 *cagA* positive cases 55.6% are in the 41-60 years age group and most (66.6%) of the *cagA* positive cases found in male patients. Significant percentage (33.3 and 50) of *cagA* was found among the patients suffering from melena and hematemesis. The result of the present study by PCR-RFLP pattern analysis revealed only β genotype for *H. pylori cagA* positive strains, which were typical genotypes in strains from Western countries. Therefore, it seems that the evaluation of genetic diversity in *H. pylori*-associated *cagA* gene can be attributable to the colonial relationship and epidemiology of H. pylori in defined population.

Keywords: *Helicobacter pylori*, *cagA*, polymorphism & RFLP

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INTRODUCTION

The prevalence of *Helicobacter pylori (H. pylori)* infections is not same in different parts of the world. Recent studies reported that humans actually acquired *H. pylori infection* in the early days of their history, long before the migration of modern humans out of Africa, and the diverse distribution of *H. pylori* today is associated with waves of human migration in the past. The rate of *H.pylori* infection is high in Africa, East Asia, and South Asia; however, the incidence of gastric cancer is high in East Asia but not in South Asia or Africa; this may be explained partly by the diversity of *H. pylori* strains in these regions [1].

H. pylori associated gastritis is now a days recognized as the major cause of duodenal and gastric ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma. The reasons for such a clinically diverse outcome of infection may include host and environmental factors as well as differences in the prevalence or expression of bacterial virulence factor[2]. The individual *H. pylori* isolates demonstrate a high level of genomic diversity as defined by different techniques. Genomic differences may affect virulence factors, altering their function and antigenicity. Antigenic variation of certain gene products may represent an immune escape mechanism for *H. pylori* strains in the host organism. [3]

A large number of studies have attempted to identify virulence markers genotypes in *H. pylori* allowing the disease outcome of an infection to be predicted. These studies were mainly based on analysis of *cagA*[4], *vacA*[5], and urease gene[6]. According to the literature, virulence cytotoxin *cagA* producing strains are more common among patients with a variety of clinical symptoms of gastritis, gastric ulcer, duodenal ulcer, and reflux esophagitis.[7] However, discrepancies on the association of different genotypes with increased virulence and ulcer and nonulcer disease or gastric carcinoma development, have been described in reports from diverse geographical regions worldwide.[8]

Bangladesh is a developing country located in Southeast Asia. However, according to historical and emigrational evidence, the Bangladeshi are more closely related to people from South Asia than people from East Asia. H. pylori infection is common in Bangladesh approximately more than 70% of individuals infected. The prevalence of gastric cancer in Bangladesh is not as high as that in Japan, China, Vietnam or Korea. Therefore, the present study was aimed at investigating cagA status present in H. pylori isolated from Bangladesh patients, as well as the relevance of genotyping these virulence factor to define these strain genetic diversity and correlation between genotypes and clinical outcomes by PCR-RFLP.

MATERIALS AND METHOD

This observational prospective study having both descriptive and analytical components, conducted in the Gastroenterology Centre, Combined Military Hospital (CMH), Dhaka-1212 & Department of Microbiology & Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Shahbagh, Dhaka-1000, Bangladesh. The study was carried out during the period of 01 July, 2013 to 31 December, 2013.

Patients with abdominal discomfort and pain for more than one month with *H. pylori* antibody test positive without any history of consuming alcohol and taking NSAIDs during this period were enrolled as study cases from the Gastroenterology center of CMH. General exclusion criteria for patients' recruitment to the study were previous attempts to eradicate *H. pylori*, use of antibiotics, proton pump inhibitors or bismuth compounds within the last 4 weeks to endoscopy, and previous gastric surgery. All patients were unrelated and Bangladeshi in origin. Written informed consent was obtained from all patients under the study, explaining the risk and benefits and objectives of the study before sampling. Clinical examination and Endoscopic findings were recorded in a predesigned data record form. Three biopsy specimens were collected during the endoscopic procedure from the greater curvature of the body of stomach, incisura angularis and antrum with positive endoscopic evidence of gastritis. Rapid urease test performed with one of the tissue specimens and results were recorded. One tissue specimen fixed in 10% buffered formalin and send to Armed Forces Institute of Pathology (AFIP), Dhaka and the diagnosis of *H. pylori* infection and confirmation of gastric disease by histopathological examination by pathologist. Another biopsy specimen preserved in sterile normal saline and send to the Department of Microbiology and Immunology, BSMMU for *cagA* gene detection and genotyping by PCR - RFLP.

DNA extraction and PCR examination:

The tissues were homogenized by mincing and grinding Helicobacter pylori were pelleted. The DNA was extracted with an equal volume of phenol-chloroform-isopropanol (25:25:1), precipitated with 2 volume of 97% ice cold ethanol followed by washing with 70% ethanol, and re-dissolved in 50 µl of TE buffer, as described in detail elsewhere[9] and used for PCR. The primer sequences were obtained from TIB MOLBIOL Syntheselabor GmbH (Berlin, Germany). The *cagA* gene forward primer 5'-3' sequence AGTAAGGAGAAACAATGA and reverse primer 5'-3' sequence AATAAGCCTTAGAGTCTTTTTGGAAATC were used for 1,320 bp PCR product. All PCR mixtures contained 1 x PCR buffer, 200 µM each deoxynucleoside

triphosphate, 25 pmol of each primer, 1.5 mM $MgCl_2$, 5U of Taq polymerase and 10 µl of DNA extracted from H. pylori. Amplification were carried out in a gradient thermal cycle (Eppendorf, Germany). Individual PCR products were electrophoresed on agarose gels, stained with ethidium bromide, and were photographed.

The PCR-RFLP analysis:

The PCR amplified cagA fragments were digested with Hinfl for 4 hours at 37C in the appropriate buffer recommended by the supplier (MBI, Fermentas, Lithuania). The digests were analyzed by electrophoresis in a 2% agarose gel with 1 x EDTA buffer followed by ethidium bromide staining.

Statistical analysis:

SPSS 23 was used for statistical evaluation and data derived from the results of the procedures mentioned above. An amount of <0.05 was accepted for P value as statistically significant.

RESULTS AND DISCUSSION

Anti-*H. pylori* IgG serology positive in all of the patients, as positive serology was considered as the selection criteria of the study cases. Rapid urease test were found to be positive in 35.29% cases and histopathological examination for *H. pylori* were positive in 19.6% cases. Among the histopathology positive *H. pylori* cases 90% were positive for *cagA* gene by PCR and almost 100% *cagA* were beta genotype by PCR-RFLP. No alpha genotype of *cagA* was found. (Table 1)

Table 01: Results of different laboratory tests for *H. pylori* detection and their *cagA* status (N=51)

Tests	Positive	cagA positive	cagA genotypes	
			α	β
Serology	51 (100)			
Histopathology	10 (19.6)	09 (90)	00	09 (100)*
Rapid urease test	18 (35.29)			

Figures within the parenthesis indicate percentage

*Statistically significant

Mean age of the patients were 46.9 ± 14.2 years starting from 22to 76 years. Out of 51 patients 39(76.47%) were male and 23.52% were female. According to the age group distribution, 22 (43.13%) were in 41-60 years age group and 39.21%and 17.64% were in 20-40 years and 61-80 years age group respectively. Among the 09 *cagA* positive cases 55.6% are in the 41-60 years age group and most (66.6%) of the *cagA* positive cases found in male patients. (Table 2)

Table 02: Age and sex distribution of the study cases and their *cagA* status (N=52)

cagA	Age group (Years)			Sex	
	20-40	41-60	61-80	Male	Female
cagA negative	17 (40.5)	17 (40.5)	8 (19)	33 (78.57)	10 (23.8)
cagA positive	3 (33.3)	5 (55.6)*	1 (11.1)	06 (66.6)*	02 (33.3)
Total	20 (39.21)	22 (43.13)	9 (17.64)	39 (76.47)*	12 (23.52)

Figures within the parenthesis indicate percentage

*Statistically significant

Most (50.98%) of the patients were suffering from upper abdominal pain and 7.69% of *cagA* gene were found in the patients suffering from upper abdominal pain. Dyspeptic patients were 33.3% and 11.76% patients were suffering from melena. Significant percentage (33.3 and 50) of *cagA* was found among the patients suffering from melena and hematemesis. (Table 3)

Table 03: Clinical symptoms of the study cases and their association with the *H. pylori* and *cagA* (N=51)

Clinical findings	Number	*cagA* positive
Dyspepsia	17 (33.3)	04 (23.52)
Upper abdominal pain	26 (50.98)*	02 (7.69)
Melena	06 (11.76)	02 (33.3)*
Hematemesis	02 (3.92)	01 (50)*

Figures within the parenthesis indicate percentage

*Statistically significant

During endoscopic procedure, 54.9% showed normal findings of upper gastro-intestinal tract. Erythema with edema in body and antrum was found in 21.56 % cases, erythema with hemorrhage in body & antrum in 7.84% cases and erythema in body and antrum were found in 13.725 cases. Significant percentage (66.6) of *cagA* were found in the patients showed erythema with edema in body and antrum during endoscopic procedure. (Table 4)

Table 04: Endoscopic findings of study cases and their *cagA* status (N=51)

Endoscopic findings	Number	*cagA* positive
Erythema with hemorrhage in body & antrum	04 (7.84)	02 (22.2)
Erythema in body & antrum	07 (13.72)	01 (11.1)
Erythema with edema in body & antrum	11 (21.56)	06 (66.6)*
Normal findings	28 (54.90)*	00

Figures within the parenthesis indicate percentage

*Statistically significant

Histopathological examinations done only with the tissues from 23 patients collected from upper GIT during endoscopic procedure in case of any abnormality found. The result of histopathological examinations showed chronic gastritis with *H. pylori* in 39.13% cases and

cagA was detected in all cases. Gastric adenocarcinoma with *H. pylori* was detected in only one case with detection of *cagA*. (Table 5)

Table 05: Histopathologic findings of study cases with positive endoscopic findings and their *cagA* status (N=23)

Histopathologic findings	Number	*cagA* positive
Chronic gastritis without *H. pylori*	13 (56.52)*	00
Chronic gastritis with *H. pylori*	09 (39.13)	09 (39.13)*
Gastric adenocarcinoma with *H. pylori*	01 (4.34)	00

Figures within the parenthesis indicate percentage

*Statistically significant

DISCUSSION:

Helicobactor pylori (*H pylori*) is one of the commonest and most ancient chronic bacterial infection of mankind. Like other developing countries, the prevalence of *H pylori*is very high in Bangladesh. More than 80.0% children become infected with *H pylori* by the age of 6-9 years[10]and 92.0% adult population are seropositive for *H pylori* in Bangladesh[11].

In the present study, anti-*H. pylori* IgG serology positive in all of the patients, as positive serology was considered as the selection criteria of the study cases. Serologic test detects *H pylori* specific IgG antibodies in serum of patients. Positive serological test means either the patient is infected at the time of the test, or the patient was once infected but, by the time, the test is done infection has resolved or the test is detecting non-specific cross-reacting antibodies. Diagnostic accuracy of various commercial serology-kits differs substantially. This is because of the antigenic differences of strains of *H pylori* in different group of population in the world. So, serology assays using antigen from one part of the world may not be appropriate when applied to another population. In a study in Bangladesh, the sensitivity, specificity, positive predictive value and negative predictive value of ELISA based serologic test were found to be 97.0%, 43.0%, 83.0% and 82.0%respectively[12].

Rapid urease test were found to be positive in 35.29% cases and histopathological examination for *H. pylori* were positive in 19.6% cases in the present study. Invasive tests to detect *H pylori* require endoscopy and biopsy. Endoscopy of upper GIT is recommended to be done in patients aged more than 50 years, in presence of alarm features or in patients not responding to initial empiric therapy. The advantage of endoscopy is to diagnose organic disease in addition to taking biopsy specimens for histology, rapid urease testing, brush-cytology and also for culture of *H pylori*. Culture and sensitivity for *H pylori* is done only in advanced centre and not commercially available in Bangladesh. Rapid unease tests (RUT) are easy, cheap and are reasonably accurate.

The sensitivity of RUT is about 90.0% to 95.0% and specificity is 95.0-100.0%[13]. The sensitivity is affected by the number of bacteria present in the biopsy and the minimum number is 10^4. Low sensitivity and specificity are also reported in patients taking proton pump inhibitors or antibiotics post-treatment, bleeding patients and in patients with achlorhydria.

During endoscopic procedure, 54.9% showed normal findings of upper gastro-intestinal tract. In this study tissue collected only from 23 patients shown gastric mucosal abnormalityduring endoscopic examination. Erythema with edema in body and antrum was found in 21.56 % cases, erythema with hemorrhage in body & antrum in 7.84% cases and erythema in body and antrum were found in 13.725 cases. Significant percentage (66.6) of *cagA* were found in the patients showed erythema with edema in body and antrum during endoscopic procedure. For histopathological diagnosis, adequate exposure and training in the detection of *H pylori* are very much essential for histopathologists. Tissue collected during the time of endoscopy may didn't contain the *Helicobacter pylori* homogenously in it. The result of histopathological examinations showed chronic gastritis with *H. pylori* in 39.13% cases and *cagA* was detected in all cases. Gastric adenocarcinoma with *H. pylori* was detected in only one case without detection of *cagA*. In this particular case of adenocarcinoma may be due to other causes than helicobacter induced pathology.

In this study histopathology examination positive for *Helicobacter pylori* considered as the gold standard for the diagnosis of tissue invasion. Among the histopathology positive *H. pylori* cases 90% were positive for *cagA* gene by PCR and by using *Hinfl* restriction enzyme, we found only β genotype by RFLP without any α genotype. This finding is in accordance with Saribasaket al[5]study stating that they could find only one genotype for *H. pylori cagA* positive strains, which were typical genotypes in strains from Western countries. Therefore, it seems that the evaluation of genetic diversity in *H. pylori*-associated *cagA* gene can be attributable to the colonial relationship and epidemiology of H. pylori in defined population.

Most (50.98%) of the patients in the preset study were suffering from upper abdominal pain and 7.69% of *cagA* gene were found in the patients suffering from upper abdominal pain. Dyspeptic patients were 33.3% and 11.76% patients were suffering from melena. Significant percentage (33.3 and 50) of *cagA* was found among the patients suffering from melena and hematemesis. *H pylori* infection is a common and curable cause of dyspepsia, peptic ulcer diseases and few upper GI malignancies. Approximately 17.0% of infected patients develop peptic ulcer, and one quarter of such patients experience an ulcer complication[14]. In a community based endoscopic survey

among adult population, the prevalence of peptic ulcer disease was found to be 15.6% in Bangladesh[15].

CONCLUSION

The result of the present study by PCR-RFLP pattern analysis revealed only β genotype for *H. pylori cagA* positive strains, which were typical genotypes in strains from Western countries. Therefore, it seems that the evaluation of genetic diversity in *H. pylori*-associated *cagA* gene can be attributable to the colonial relationship and epidemiology of H. pylori in defined population. One of the important limitations of this study was that, very small sample size which may not reflect the scenario of the large population group. Further study with more sample size to detect all of the virulence gene and their genotyping is recommended.

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REFERENCES

1. Yamaoka, Y. M. Kato, and M. Asaka. Geographic differences in gastric cancer incidence can be explained by differences between *Helicobacter pylori* strains. Intern. Med 2008; 47: 1077–1083.
2. Malaty HM, Engstrand L, Pedersen NL, Graham DY. *Helicobacter pylori* infection: genetic and environmental influences. A study of twins. Ann intern Med 1994; 120: 982-986.
3. Akopyanz N, bukanow NO, Westblom TU, Berg DE. PCR-based RFLP analysis of DNA sequence diversity in the gastric pathogen *Helicobacter pylori*. Nucleic Acids Res 1992; 20: 6221-6225.
4. Atherton JC, Peek RM, Tham KT, Cover TL, Blaser MJ. Clinical and pathological importance of heterogenicity in *vacA*, the vacuolatingcytotoxin gene of *Helicobacter pylori*. Gastroenterology 1997; 112: 92-99.
5. Saribasak H, Salih BA, Yamaoka Y, Sander E. Analysis of *Helicobacter pylori* Genotypes and Correlation with Clinical Outcome in turkey. J ClinMicrobiol 2004; 42: 1648-1651.
6. Olmos JA, Rios H, Higa R. Prevalence of *Helicobacter pylori* infection in Argentina: results of a nationwide epidemiological study. Argentinean Hp Epidemiological Study Group. J ClinGastroenterol 2000; 31: 33-37.

7. Yamoka Y, kodama T, Gutierrez O, Kim JG, Kashim K, Graham DY. Relationship between *Helicobacter pylori iceA, cagA* and *vacA* status and clinical outcome: studies in four different countries. J ClinMicrobiol 1999; 37: 2374-2379.
8. Covacci A, Telford JL, Giudice GD, Parsonnet J, Rappuoli R. *Helicobacter pylori* virulence and genetic geography. Science 1999; 284: 1328-1333.
9. Wilson K. Preparation of genomic DNA from bacteria. In: Ausubel FA, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JE, Struhl K, editors, Current protocols in molecular biology. New York (NY): John Wiley & Sons; 1994. P. 2.4.1-2.4.5.
10. Mahalanabis D, Rahman MM, Sarker SA, Bardhan PK, Hildebrandet P, Beglinger C, et al. *Helicobactor pylori* infection in the young in Bangladesh: Prevalence, Socioeconomic and Nutritional Aspects. International J Epidemiol 1996;25: 894-898.
11. Ahmad MM, Rahman M, Rumi MAK, et al. Prevalence of *Helicobacte rpylori* in asymptomatic population - a pilot serological study in Bangladesh. Japan J Epidemiol 1997; 7: 251-54.
12. Rahman SHZ, Azam M G, Rahman M A, Arfin MS Alam, M M, Bhuiyan T M, Ahmed N, Rahman M, Nahar S, Hassan MS. Non-invasive diagnosis of *H pylori* infection: Evaluation of serological tests with and without current infection marker CIM. World J Gastroenterol
    2008; 14(8): 1231-1236.
13. Ricci C. Diagnosis of *Helicobacter pylori*: Invasive and non-invasive tests. Best Practice & Research Clinical Gastroenterology 2007; 21: 299-313.
14. Tytgat GNJ, Lee A, Graham DY et al. The role of infectious agents in peptic ulcer disease. GastroenterolInternat 1993; 6: 76-89.
15. Hasan M, Azad khan AK, Roy PK, Aziz S, Huq KM, Ali SMK et al. Peptic Ulcer in Bangladesh. BMRC Bulletin 1987; 13: 29-42.

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