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PCR-based analysis of *Helicobacter pylori* virulent genotypes among dyspeptic patients from Chittagong, Bangladesh

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ABSTRACT

Aims: *Helicobacter pylori* is a causative agent of gastroduodenal diseases in Bangladesh as well as throughout the world. This study aimed to determine the *H. pylori cagA*, *vacA* and *iceA* virulent genotypes by PCR directly in gastric biopsies from dyspeptic patients of Chittagong, Bangladesh and evaluating the association of these genotypes with clinical manifestations.
Methodology and results: CLO (Campylobacter-Like Organism) test and Hp16s PCR (*16S rRNA* based *H. pylori* specific PCR) was performed to confirm *H. pylori* infection. Among 111 patients, *H. pylori* infection was found in 60 patients by CLO test, while Hp16s PCR revealed that 54 patients were *H. pylori* positive. PCR amplification of the *H. pylori* virulence genes was successful in 35 gastric biopsies amongst the 54 Hp16s PCR positive biopsies. The positive rates for the *cagA*, *vacAs1*, *vacAs2*, *vacAm1*, *vacAm2*, *iceA1*, *iceA2* genes were 34.3%, 71.4%, 8.6%, 62.9%, 28.6%, 20% and 11.4%, respectively. The allelic variant *vacAs1m1* had a predominant percentage with 51.4%, followed by *vacAs1m2*, *vacAs2m2* and *vacAs1m1m2* with 14.3%, 5.7% and 2.9%, respectively. Among the subtypes of *vacAs1*, only *s1a* was detected in 54.3% of biopsies while none of the cases showed the s1b and s1c genotypes. However, there was no statistically significant association (P>0.05) observed between the virulent genotypes and clinical conditions.
Conclusion, significance and impact of study: We found that *cagA*, *vacAs1m1* and *iceA1* were the most frequent *H. pylori* genotypes in severe clinical outcomes of the infection. The data in this study would provide a basis for understanding the diverse virulence pattern of this bacterium in Bangladeshi dyspeptic patients.

Keywords: *Helicobacter pylori,* gastroduodenal diseases, gastric biopsies, genotypes, PCR.

INTRODUCTION

Helicobacter pylori is a common pathogenic bacterium that chronically infects gastric surfaces of over 50% of human population worldwide (Garza-Gonzalez *et al.*, 2014). It is associated with a number of gastroduodenal diseases including acute and chronic active gastritis, peptic ulcer diseases, mucosa associated lymphoid tissue (MALT) lymphoma and gastric malignancy (Suzuki *et al.*, 2012). Interestingly, even with the high *H. pylori* infection rate in some countries, the frequency of severe disease outcomes is considerably lower than other populations. Apart from the host gastric mucosal factors and environmental factors, the genetic heterogeneity in the *H. pylori* virulence factors contributes to the varied clinical outcomes of *H. pylori* infection (Bani-Hani, 2002). Several virulence factors such as vacuolating cytotoxin A (*vacA*), cytotoxin-associated gene A (*cagA*), and induced by contact with epithelium A (*iceA*) gene play vital role in the development of various gastric diseases (Yamaoka, 2010; Suzuki *et al.*, 2012). The *cagA*, which may not be found in every *H. pylori* strain, is known as a marker for the 40-kb *cag* pathogenicity island (*cag*-PAI) and its expression results in an enhanced interleukin 8 production, gastric mucosal inflammation and more severe clinical manifestations of the infection (Censini *et al.*, 1996; Akopyants *et al.*, 1998). On the contrary, the *vacA* is virtually present in all *H. pylori* strains, although the VacA toxin might not be expressed in every case (Atherton *et al.*, 1995). The *vacA* gene has two types of signal regions (*s1* or *s2*), and two sorts of middle regions (*m1* or *m2*) (Aziz *et al.*, 2014). Again within the s1 region, there are three allelic forms: *s1a*, *s1b*, and *s1c* (Aziz *et al.*, 2014). Strains with the *vacAs1m1* genotype demonstrate a high cytotoxic activity and have been linked to severe clinical diseases such as peptic ulcer diseases (Atherton *et al.*,

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1997). Another important virulence marker is *iceA* (induced by contact with the epithelium), which exists in at least two allelic subtypes, *iceA1* and *iceA2* (Van Doorn *et al.*, 1998a). The *iceA1* gene is transcribed upon contact of *H. pylori* with gastric epithelium and may be associated with peptic ulcer diseases (Peek Jr *et al.*, 1997). However, subsequent studies conducted in different parts of the world have shown considerable inconsistencies in these disease associations of *H. pylori* genotypes (Yamaoka *et al.*, 1999, Van Doorn *et al.*, 1999). In Bangladesh, the rate of *H. pylori* infection among infants, children, and adults account for 61%, 84%, and 92%, respectively (Nahar *et al.*, 2004). But there is not much information available as regards to the pattern of *H. pylori* genotypes in Bangladesh, particularly in Chittagong region, the south-eastern part of the country. The determination of the frequency of *vacA, cagA*, and *iceA* genotypes in Chittagong would be of great value to understand molecular epidemiological status of *H. pylori* strain in the southeastern part of Bangladesh. Hence, this study aimed to analyse *vacA*, *cagA*, and *iceA* genotypes with their clinical manifestations in patients with gastric diseases from Chittagong, by using PCR directly in gastric biopsies from the dyspeptic patients of the region.

MATERIALS AND METHODS

Patients

Gastric biopsy specimens from 111 individuals referred to esophagogastroduodenoscopy at a hospital in Chittagong from July 2015 to November 2015 were collected. Each of the patients was informed about the objective of the study and written informed consent was taken under a protocol endorsed by the Ethical Review Committee of the Bangladesh Medical Research Council. Patients who had previous gastric surgery, who had consumed antibiotics, proton pump inhibitors (PPIs), or bismuth compounds in the last month were not included in this study. Two antral gastric biopsy specimens from each patient were taken by a specialized physician using biopsy forceps, which were cleaned with detergent and disinfected after each use. On the basis of endoscopic observations, patients were categorized as having gastritis, duodenal ulcer, gastric ulcer, or suspicion of gastric cancer which was confirmed with extra biopsies by pathologist. Immediately after collection, the biopsy specimens were transported to the laboratory in falcon tube with 5 mL 0.9% sterile saline solution.

CLO (*Campylobacter*-Like Organism) test

One antrum biopsy specimen was introduced with a sterile pipette tip into a semisolid 2% urea agar and then subjected to incubation at room temperature. Results were obtained 4 h after inoculation (Deltenre *et al.*, 1989).

DNA extraction directly from biopsies

The whole genomic DNA was isolated from the gastric biopsies by phenol/chloroform DNA extraction method as described in the literature (Ho *et al.*, 1991).

PCR assays for *H. pylori* identification and determination of genotypes

Primer sequences for PCR amplifications of the *16S rRNA* gene and for the genotypes *cagA, vacA* and *iceA* were designed based on published papers (Ho *et al.*, 1991; Van Doorn *et al.*, 1998b; Lee *et al.*, 2004) with a modification of PCR mixtures and annealing temperatures (Table 1). Each PCR was carried out in a total volume of 25 µL containing 3-4 µL (approximately 350 ng) of genomic DNA from gastric biopsies, 3 µL (30 pmol) each of forward and reverse primers, 12.5 µL of 2X master mix (Promega) and required volume of nuclease free water. The PCR cycles were carried out in a Qantarus Thermal cycler (Model Q-cycler, UK).

Agarose gel electrophoresis

The amplified PCR products of the virulence genes were subjected to electrophoresis in a 1.5% agarose gel, stained with ethidium bromide and bands were observed under ultraviolet light in a gel documentation system (WGD-30, WiseDoc, Seoul, Korea).

Data analysis

Statistical analysis was performed by SPSS version 16. The Pearson X^2 test was done to analyze the association between individual genotypes and disease outcomes such as gastritis, gastric ulcer, duodenal ulcer, gastric cancer and normal endoscopic finding.

RESULTS

Out of 111 subjects enrolled in this study, 55 (49.5%) were males, and 56 (50.5%) were females with a mean age 42.5 ±13.9 years (range 15 to 73 years old). Twenty patients (18%) had gastric ulcers, 4 (3.6%) had duodenal ulcers, 74 (66.7%) had gastritis, 5 (4.5%) had gastric cancer and 8 patients (7.2%) were with normal endoscopic finding. Among the 111 patients, 60 were positive by CLO test, 54 (48.65%) were positive by PCR and 40 (36.04%) were positive by both PCR and CLO test. Since the CLO test was performed for preliminary *H. pylori* detection, the biopsies positive only for CLO test were not used for PCR amplification of the *H. pylori* virulence genes. However, all of the fifty-four Hp16s PCR positive biopsies were investigated for the presence of c*agA*, *vacA* and *iceA* genotypes. The biopsies that were PCR positive for at least one or more of the genes comprised of 35 (63%) biopsies while 19 (37%) biopsies were negative for all genes. Therefore, further analysis was done in these 35 biopsies.

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Table 1: Primer sequences and annealing temperatures used in this study.

Genes	Primer sequence (5´- 3´)	Annealing temperature (°C)	PCR product (bp)	Ref.
H.pylori 16S rRNA	CTG GAG AGA CTA AGC CCT CC ATT ACT GAC GCT GAT TGT GC	55	109	Ho *et al.,* 1991
cagA	GAT AAC AGG CAA GCT TTT GAG G CTG CAA AAG ATT GTT TGG CAG A	55	349	Van Doorn *et al.,* 1998b
vacA s1/s2	ATG GAA ATA CAA CAA ACA CAC CTG CTT GAA TGC GCC AAA C	52	259 (s1) 286 (s2)	Van Doorn *et al.,* 1998b
vacA s1a	GTC AGC ATC ACA CCG CAA C CTG CTT GAA TGC GCC AAA C	52	190	Van Doorn *et al.,* 1998b
vacA s1b	AGC GCC ATA CCG CAA GAG CTG CTT GAA TGC GCC AAA C	55	187	Van Doorn *et al.,* 1998b
vacA s1c	TTA GTT TCT CTC GCT TTA GTR GGG YT CTG CTT GAA TGC GCC AAA C	55	220	Lee *et al.,* 2004
vacA m1/m2	CAA TCT GTC CAA TCA AGC GAG GCG TCT AAA TAA TTC CAA GG	49.3	570 (m1) 645 (m2)	Van Doorn *et al.,* 1998b
iceA1	GTG TTT TTA ACC AAA GTA TC CTA TAG CCA STY TCT TTG CA	46	247	Van Doorn *et al.,* 1998b
iceA2	GTT GGG TAT ATC ACA ATT TAT TTR CCC TAT TTT CTA GTA GGT	46°	229 or 334	Van Doorn *et al.,* 1998b

190 bp) subtype of *vacAs1* was detected (Figure 2) in 19/35 (54.3%) biopsies while none of the biopsies showed the *s1b* and the *s1c* subtypes. With regard to the m region of *vacA*, 31 (88.6%) of 35 biopsies were positive for it; *m1* allele was predominantly detected in 22/35 (62.9%), compared to *m2* allele identified (Figure 3) in 10/35 (28.6%) biopsies. However, both *m1* and *m2* products, suggesting a co-infection with two different strains, were observed only in one patient (2.9%). A combination of the *vacA*s, and *m* regions was determined in 26/35 (74.3%) of the biopsies. The allele s1m1 was the most common *vacA* allelic combination accounting for 51.4% (18/35), followed by *s1m2*, *s2m2* and *s1m1m2* with 14.3% (5/35), 5.7% (2/35) and 2.9% (1/35), respectively. However, none of the biopsies showed the *vacAs2m1* genotype. Moreover, the incomplete *vacA* where either *vacAs* or *vacAm* regions were not detected constituted 7/35 (20%) while 2/35 (5.7%) failed to amplify for *vacA* gene.

The *cagA* gene was amplified (Figure 4) in 13/35 (37.1%) of the biopsies. On the other hand, only 11 (31.4%) biopsies represented a single *iceA* allele; *iceA1* was detected (Figure 5) in 7 (20%) and *iceA2* (Figure 6) in 4 (11.4%) biopsies. Only one biopsy was identified with both *iceA* alleles (2.9%). Interestingly, a probable deletion in the *iceA1* (a fragment of less than 200 bp instead of 247 bp) as found by Rahman *et al.* (2003) was found in two cases.

H. pylori Genotypes and Clinical Association

After excluding one mixed, seven incomplete and two unamplified *vacA* genotype samples from 35 biopsies (28.6%), altogether, 25 biopsies were considered for assessing the relationship between *vacA* genotypes and

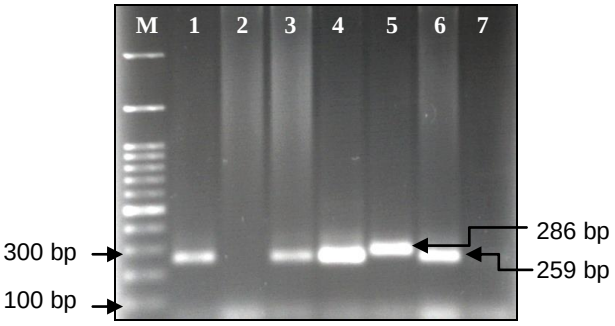

Figure 1: PCR Detection of *vacAs* (signal) region. Lane M is a 100 bp ladder; lanes 1, 3, 4, 6 demonstrated the presence of *s1* (259 bp); lane 5 was *s2* (289 bp) positive and lanes 2 and 7 represented negative samples.

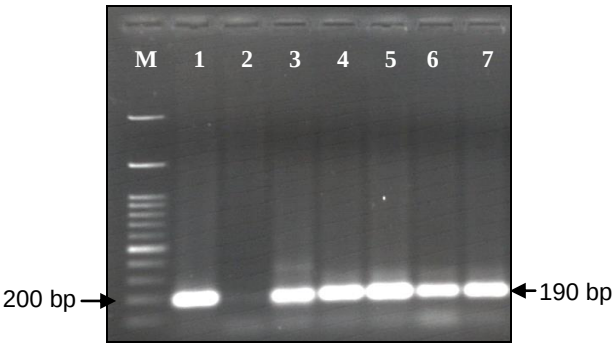

Figure 2: PCR Detection of *vacAs1a* gene. Lane M is a 100 bp ladder; lanes 1 and 3-7 showing the PCR amplification of *s1a* (190 bp); lane 2 represented *s1a* negative sample.

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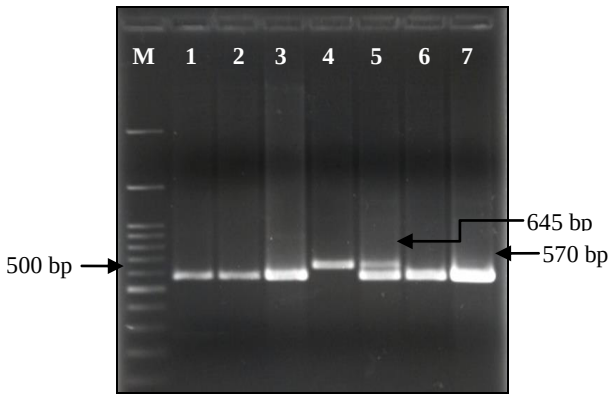

Figure 3: PCR Detection of *vacAm* (middle) region. Lane M is a 100 bp ladder; lanes 1, 2, 3, 6 and 7 are *m1* (570 bp) positive; lanes 4 showed the presence of *m2* (645 bp); lane 5 was both *m1* and *m2* positive.

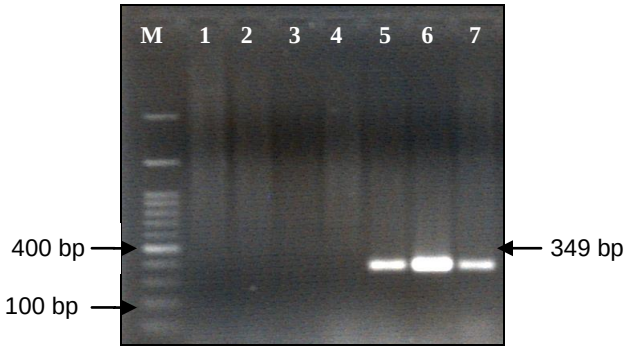

Figure 4: PCR Detection of *cagA* gene. Lane M is a 100 bp ladder; lanes 5, 6, 7 showed PCR products (349 bp) of *cagA* genes; other lanes are *cagA* negative.

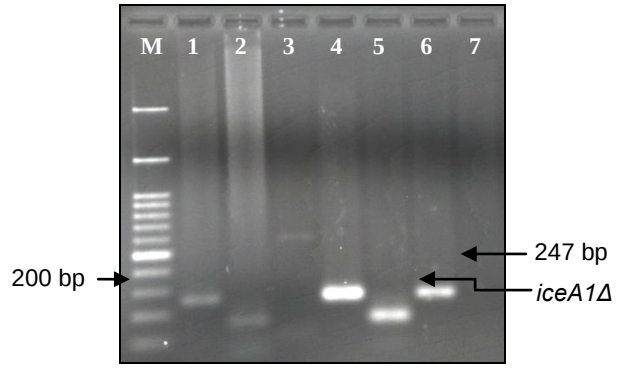

Figure 5: PCR Detection of *iceA1* genotype. Lane M is a 100 bp ladder, lanes 1, 4 and 6 showed the PCR amplification of 247 bp of *iceA1*; lanes 2 and 5 demonstrated a probable deletion in *iceA1*; lanes 3 and 7 were *iceA1* negative.

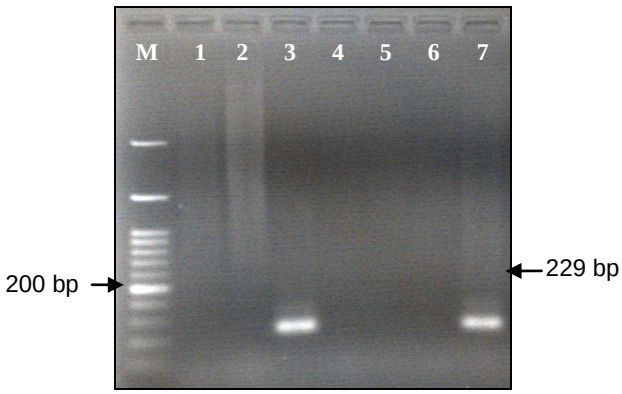

Figure 6: PCR Detection of *iceA2* genotype. Lane M is a 100 bp ladder; lanes 3 and 7 represented PCR amplification of 229 bp *iceA2* and other lanes showed the absence of *iceA2*.

clinical presentation. Of the 25 biopsies, all the 6 patients diagnosed with gastric ulcer and the only one patient with duodenal ulcer were found to have the toxigenic *s1m1* genotype while in gastritis and normal endoscopic findings the percentage of *s1m1* was 60% (9/15) and 66.7% (2/3) respectively. On the other hand, the less toxigenic *s1m2* was found merely in the patients with gastritis that accounted for 33.3% (5/15), while the non-toxigenic *s2m2* was present in 6.3% (1/15) and 33.3% (1/3) of the patients with gastritis and normal endoscopic findings respectively (Figure 7a). Despite the fact that the toxigenic *s1m1* allele of the *vacA* gene was more frequently identified in patients with gastric and duodenal ulcer, no statistically significant association ($P > 0.05$) was observed.

In this study, the correlation between *cagA* and disease outcomes was evaluated, and although none of the patients with gastric cancer showed *cagA* genotype, it was found to be prevalent more frequently among the patients with gastric ulcer and duodenal ulcer than those with gastritis: gastric ulcers 50% (4/8); duodenal ulcers 50% (1/2); gastritis 40% (8/20) (Figure 7b). However, no statistically significant association was found between *cagA* genotype and gastroduodenal diseases ($P > 0.05$).

In terms of assessing the relationship between *iceA* genotypes and gastroduodenal diseases, only 11 biopsies were considered excluding one mixed (that was from antral ulcer) and 23 unamplified *iceA* genotype samples from 35 biopsies. The *iceA1* gene was found to be present in all of the four gastroduodenal diseases while the *iceA2* gene was observed only in two patients with gastritis and one with gastric ulcer and another one with normal endoscopic finding (Figure 7c) while the relationship between *iceA* genotypes and the disease outcomes was not statistically significant.

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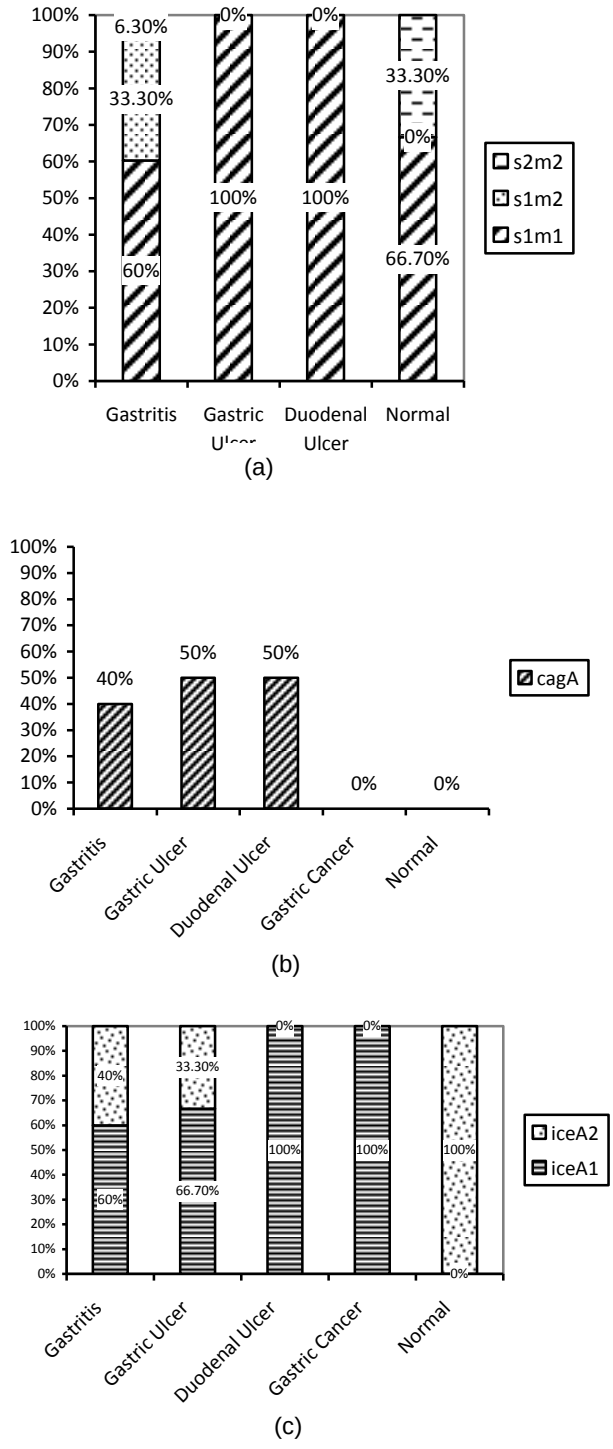

Figure 7: *vacA* (a), *cagA* (b), and *iceA* (c), genotypes in relation to gastroduodenal diseases. *cagA*, *vacAs1m1* and *iceA1* were the most predominant virulent genotypes in severe clinical outcomes of the infection.

DISCUSSION

In the present study, *H. pylori* infection was confirmed by Hp16s PCR and CLO test which are rapid, efficient and reliable methods for diagnosing *H. pylori* (Garza-Gonzalez *et al.*, 2014). Though culture is considered to be the gold standard method for detecting bacterial infection, it was not used in our study because for culturing a slow growing organism like *H. pylori*, it takes several days to obtain results. Besides, culture relies very much on infrastructure conditions and it is greatly affected by frequent power outage in developing countries like Bangladesh. However, PCR for detecting *H. pylori* genotypes was carried out in 54 Hp16s PCR positive biopsies. The biopsies, which were only CLO test positive but Hp16s PCR negative, were not considered for genotyping because the CLO test was performed for preliminary *H. pylori* identification.

PCR amplification of the *H. pylori* virulence genes was successful in 35 gastric biopsies out of 54 Hp16s PCR positive biopsies. Inability to identify the virulent genes in all of the Hp16s PCR-positive biopsies could be due to the small amount of gastric tissue obtained by a single biopsy or to the presence of a lower number of *H. pylori* on gastric mucosa in some biopsies. Moreover, another important fact is that the *16S rRNA* gene PCR had a slightly superior level of analytical sensitivity when compared with the other primers (Rudi *et al.*, 2000). Therefore, an increase in the number of biopsies may improve the success rate of amplifying *cagA*, *vacA* and *iceA* genotypes by PCR.

The *vacA* genotypes of *H. pylori* are considerably diverse in different geographic regions. In our study, we predominantly found the toxigenic *vacA* genotypes of *s1* and *m1*, while the non-toxigenic *s2* and *m2* were found in low frequency. Moreover, our study for the first time in Bangladesh detected *s1a* subtype of *vacAs1* in 19/35 (54.3%) of biopsies but none of the cases harbor the *s1b* and *s1c* subtypes of *vacAs1*. Overall, our findings are similar to a report from India where *vacAs1a* and *vacAm1* were found to be predominant (Mishra *et al.*, 2002) while in contrast to a study from Pakistan that reported *s1b* and *m2* to be more prevalent *vacA* genes in their population (Ahmad *et al.*, 2009). Furthermore, in this study, the combined *vacA* genotypes of different alleles were assessed in relation to clinical presentations. Despite the fact that the toxigenic *s1m1* allele of the *vacA* gene was more frequently identified in patients with gastric ulcer and duodenal ulcer, no statistically significant correlation ($P > 0.05$) was observed and this finding is in agreement with several studies from India (Mishra *et al.*, 2002; Chattopadhyay *et al.*, 2002).

The *cagA* results of 37.1% in this study are similar to those obtained in Pakistan of 24.0% (Ahmad *et al.*, 2009). However, this is amazingly low *cagA* prevalence when compared with previous study of our country (95%) (Sarker *et al.*, 2004), and with studies from India (96%), China (86%) and Iran (Qiao *et al.*, 2003; Kamali-Sarvestani *et al.*, 2006; Arachchi *et al.*, 2007). There are different possible explanations for this finding. First, *cagA*

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is more common in *H. pylori* infection associated with peptic ulcer or gastric carcinoma. In our study, 73% of patients had non-ulcer dyspepsia (67% gastritis and 6% normal) and 23% had peptic ulcer (19% gastric ulcer and 4% duodenal ulcer) and only 4% had gastric cancer. Van Doorn *et al.* (1998a) also presented a prevalence of *cagA*-positive strains of only 35.7 % in Egypt, where most of the isolates were from non-ulcer patients. Another possible explanation is that there might be a diversity of the *cagA* status in Bangladesh, which is similar to what reported in most of Asian countries, such as in Thailand, Malaysia or China (Sahara *et al.*, 2012; Aziz *et al.*, 2014). However, *cagA* was found to be prevalent more frequently among the patients with gastric ulcer and duodenal ulcer than those with gastritis but the association was not statistically significant and this finding is consistent with several reports from some Asian countries (Maeda *et al.*, 1998; Kim *et al.*, 2001; Zhou *et al.*, 2004).

To our knowledge, this is the first ever study in Bangladesh to determine *iceA* genotypes directly from gastric biopsies by PCR. Though we succeeded in amplifying *iceA* genes only in 12/35 (34.3%) of biopsies, our finding agrees with Secka *et al.* (2011) that the PCR amplification of *iceA* gene directly from gastric biopsies is relatively poor. However, amongst the *iceA*-positive *H. pylori* infected patients in our study, *iceA1* gene was found more frequently than the *iceA2* (*iceA1* 58.3%, *iceA2* 33.3%) and an *iceA1/iceA2* mixed genotype was observed as well. Our results showed different *iceA* frequency rates when compared to a previous report (Rahman *et al.*, 2003) which showed *iceA1* and *iceA2* genotypes to be almost equally distributed in Bangladeshi dyspeptic patients. However, this finding agrees with many other studies that have shown the *iceA1* allele more frequently found than *iceA2* allele in Indian, Chinese, Japanese and Korean patients (Van Doorn *et al.*, 1998a; Kim *et al.*, 2001; Chattopadhyay *et al.*, 2002; Han *et al.*, 2004); *iceA2* has been found to be predominant among European and American patients (Yamaoka *et al.*, 1999). Moreover, similar to the U.S. and European strains (Van Doorn *et al.*, 1999), the prevalence of *iceA1* allele was higher among cases with peptic ulcer and gastric cancer compared to cases with gastritis and normal endoscopic finding, but like for the *cagA* and *vacA* genotypes, this effect did not persist in statistical analysis.

CONCLUSION

In conclusion**,** this study for the first time in south-eastern Bangladesh identified *H. pylori* virulent genotypes directly in gastric biopsies by PCR and then examined the association of these genotypes with clinical presentations. We found that *cagA*, *vacAs1m1* and *iceA1* were the most predominant virulent genotypes in severe clinical outcomes of the infection. The data in this study would provide a baseline framework for future detailed investigations to understand the diversity of *H. pylori* genotypes among Bangladeshi dyspeptic patients.

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REFERENCES

Ahmad, T., Sohail, K., Rizwan, M., Mukhtar, M., Bilal, R. and Khanum, A. (2009). Prevalence of *Helicobacter pylori* pathogenicity-associated cagA and vacA genotypes among Pakistani dyspeptic patients. *FEMS Immunology and Medical Microbiology* **55, 34-38.** DOI: 10.1111/j.1574-695X.2008.00492.x.

Akopyants, N. S., Clifton, S. W., Kersulyte, D., Crabtree, J. E., Youree, B. E., Reece, C., Bukanov, N. O., Drazek, E. S., Roe, B. A. and Berg, D. E. (1998). Analyses of the cag pathogenicity island of *Helicobacter pylori*. *Molecular Microbiology* **28, 37-53.** DOI: 10.1046/j.1365-2958.1998.00770.x.

Arachchi, H., Kalra, V., Lal, B., Bhatia, V., Baba, C., Chakravarthy, S., Rohatgi, S., Sarma, P. M., Mishra, V. and Das, B. (2007). Prevalence of duodenal ulcer-promoting gene (dupA) of *Helicobacter pylori* in patients with duodenal ulcer in North Indian population. *Helicobacter* **12, 591-597.** DOI: 10.1111/j.1523-5378.2007.00557.x.

Atherton, J. C., Cao, P., Peek, R. M., Tummuru, M. K., Blaser, M. J. and Cover, T. L. (1995). Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori* association of specific vacA types with cytotoxin production and peptic ulceration. *Journal of Biological Chemistry* **270, 17771-17777.** DOI: 10.1074/jbc.270.30.17771.

Atherton, J., Peek, R., Tham, K., Cover, T. and Blaser, M. (1997). Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of *Helicobacter pylor*i. *Gastroenterology* **112, 92-99.** DOI: 10.1016/S0016-5085(97)70223-3.

Aziz, F., Chen, X., Yang, X. and Yan, Q. (2014). Prevalence and correlation with clinical diseases of *Helicobacter pylori* cagA and vacA genotype among gastric patients from Northeast China. *BioMed Research International* **2014, Article ID: 142980.**

Bani-hani, K. E. (2002). The current status of *Helicobacter pylori*. *Saudi Medical Journal* **23, 379-383.** PMID: 11953759.

Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., rappuoli, R. and Covacci, A. (1996). cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proceedings of the National Academy of Sciences* **93, 14648-14653.** DOI: 10.1073/pnas.93.25.14648.

Chattopadhyay, S., Datta, S., Chowdhury, A., Chowdhury, S., Mukhopadhyay, A. K., Rajendran,

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

K., Bhattacharya, S., Berg, D. E. and Nair, G. B. (2002). Virulence genes in *Helicobacter pylori* strains from West Bengal residents with overt *H. pylori*-associated disease and healthy volunteers. *Journal of Clinical Microbiology* **40, 2622-2625.** DOI: 10.1128/JCM.40.7.2622-2625.2002.

Deltenre, M., Glupczynski, Y., Prez, C. D., Nyst, J., Burette, A., Labbé, M., Jonas, C. and Dekoster, E. (1989). The reliability of urease tests, histology and culture in the diagnosis of *Campylobacter pylori* infection. *Scandinavian Journal of Gastroenterology* **24, 19-24.** PMID: 2479086.

Garza-González, E., Perez-Perez, G. I., Maldonado-Garza, H. J., and Bosques-Padilla, F. J. (2014). A review of *Helicobacter pylori* diagnosis, treatment, and methods to detect eradication. *World Journal of Gastroenterology* **20, 1438-1449**. DOI: 10.3748/wjg.v20.i6.1438.

Han, Y. H., Liu, W. Z., Zhu, H. Y. and Xiao, S. D. (2004). Clinical relevance of iceA and babA2 genotypes of *Helicobacter pylori* in a Shanghai population. *Chinese Journal of Digestive Diseases* **5, 181-185.** DOI: 10.1111/j.1443-9573.2004.00175.x.

Ho, S. A., Hoyle, J., Lewis, F., Secker, A., Cross, D., Mapstone, N., Dixon, M., Wyatt, J., Tompkins, D. and Taylor, G. (1991). Direct polymerase chain reaction test for detection of *Helicobacter pylori* in humans and animals. *Journal of Clinical Microbiology* **29, 2543-2549.** PMID: 1723072.

Kamali-sarvestani, E., Bazargani, A., Masoudian, M., Lankarani, K., Taghavi, A. R. and Saberifiroozi, M. (2006). Association of *H pylori cagA* and *vacA* genotypes and IL-8 gene polymorphisms with clinical outcome of infection in Iranian patients with gastrointestinal diseases. *World Journal of Gastroenterology* **12, 5205-5210.** DOI: 10.3748/wjg.v12.i32.5205.

Kim, S. Y., Woo, C. W., Lee, Y. M., Son, B. R., Kim, J. W., Chae, H. B., Youn, S. J. and Park, S. M. (2001). Genotyping *CagA*, *VacA* subtype, *IceA1*, and *BabA* of *Helicobacter pylori* isolates from Korean patients, and their association with gastroduodenal diseases. *Journal of Korean Medical Science* **16, 579-584.** DOI:10.3346/jkms.2001.16.5.579.

Lee, J. H., Choe, Y. H., Jeon, B. H., Oh, Y. J., Kim, J. J., Rhee, J. C., Kim, P. S. and Choi, M. S. (2004). Genotypes of the *Helicobacter pylori vac*A signal sequence differ with age in Korea. *Helicobacter* **9, 54-58.** DOI: 10.1111/j.1083-4389.2004.00198.x.

Maeda, S., Ogura, K., Yoshida, H., Kanai, F., Ikenoue, T., Kato, N., Shiratori, Y. and Omata, M. (1998). Major virulence factors, VacA and CagA, are commonly positive in *Helicobacter pylori* isolates in Japan. *Gut* **42, 338-343.** DOI :10.1136/gut.42.3.338.

Mishra, K., Srivastava, S., Dwivedi, P., Prasad, K. and Ayyagari, A. (2002). Genotypes of *Helicobacter pylori* isolated from various acid peptic diseases in and around Lucknow. *Current Science* **83, 749-754.**

Nahar, S., Mukhopadhyay, A. K., Khan, R., Ahmad, M. M., Datta, S., Chattopadhyay, S., Dhar, S. C., Sarker, S. A., Engstrand, L. and Berg, D. E. (2004). Antimicrobial susceptibility of *Helicobacter pylori* strains isolated in Bangladesh. *Journal of Clinical Microbiology* **42, 4856-4858.** DOI: 10.1128/JCM.42.10.4856-4858.2004.

Peek jr, R. M., Thompson, S. A., Donahue, J. P., Tham, K. T., Atherton, J. C., Blaser, M. J. and Miller, G. G. (1997). Adherence to gastric epithelial cells induces expression of a *Helicobacter pylori* gene, *iceA*, that is associated with clinical outcome. *Proceedings of the Association of American Physicians* **110, 531-544.** PMID:9824536.

Qiao, W., Hu, J. L., Xiao, B., Wu, K. C., Peng, D. R., Atherton, J. C. and Xue, H. (2003). *cagA* and *vacA* genotype of *Helicobacter pylori* associated with gastric diseases in Xi'an area. *World journal of gastroenterology* **9, 1762-1766.** DOI:10.3748/wjg.v9.i8.1762.

Rahman, M., Mukhopadhyay, A. K., Nahar, S., Datta, S., Ahmad, M. M., Sarker, S., Masud, I. M., Engstrand, L., Albert, M. J. and Nair, G. B. (2003). DNA-level characterization of *Helicobacter pylori* strains from patients with overt disease and with benign infections in Bangladesh. *Journal of Clinical Microbiology* **41, 2008-2014.** DOI:10.1128/JCM.41.5.2008-2014.2003.

Rudi, J., Kuck, D., Rudy, A., Sieg, A., Maiwald, M. and Stremmel, W. (2000). *Helicobacter pylori vacA* genotypes and *cagA* gene in a series of 383 *H. pylori*-positive patients. *Zeitschrift fur Gastroenterologie* **38, 559-564.** DOI:10.1055/s-2000-7449.

Sahara, S., Sugimoto, M., Vilaichone, R. K., Mahachai, V., Miyajima, H., Furuta, T. and Yamaoka, Y. (2012). Role of *Helicobacter pylori cagA* EPIYA motif and *vacA* genotypes for the development of gastrointestinal diseases in Southeast Asian countries: a meta-analysis. *BMC Infectious Diseases* **12, 223.** DOI: 10.1186/1471-2334-12-223.

Sarker, S., Nahar, S., Rahman, M., Bardhan, P., Nair, G., Beglinger, C. and Gyr, N. (2004). High prevalence of cagA and vacA seropositivity in asymptomatic Bangladeshi children with *Helicobacter pylori* infection. *Acta Paediatrica* **93, 1432-1436.** DOI: 10.1111/j.1651-2227.2004.tb02624.x.

Secka, O., Antonio, M., Tapgun, M., Berg, D. E., Bottomley, C., Thomas, V., Walton, R., Corrah, T., Adegbola, R. A. and Thomas, J. E. (2011). PCR-based genotyping of *Helicobacter pylori* of Gambian children and adults directly from biopsy specimens and bacterial cultures. *Gut Pathogens* **3, 5.** DOI: 10.1186/1757-4749-3-5.

Suzuki, R., Shiota, S. and Yamaoka, Y. (2012). Molecular epidemiology, population genetics, and pathogenic role of *Helicobacter pylori*. *Infection, Genetics and Evolution* **12, 203-213.** DOI:10.1016/j.meegid.2011.12.002.

Van Doorn, L. J., Figueiredo, C., Sanna, R., Plaisier, A., Schneeberger, P., De Boer, W. and Quint, W. (1998a). Clinical relevance of the *cagA*, *vacA*, and

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

iceA status of *Helicobacter pylori*. *Gastroenterology* **115, 58-66.** DOI:10.1016/S0016-5085(98)70365-8.

Van Doorn, L. J., Figueiredo, C., Rossau, R., Jannes, G., van Asbroeck, M., Sousa, J., Carneiro, F. and Quint, W. (1998b). Typing of *Helicobacter pylori vacA* gene and detection of cagA gene by PCR and reverse hybridization. *Journal of Clinical Microbiology* **36, 1271-1276.** PMID:9574690.

Van Doorn, L. J., Figueiredo, C., Mégraud, F., Pena, S., Midolo, P., Queiroz, D. M. D. M., Carneiro, F., Vanderborght, B., Maria Da Glória, F. P. and Sanna, R. (1999). Geographic distribution of *vacA* allelic types of *Helicobacter pylori*. *Gastroenterology* **116, 823-830.** DOI:10.1016/S0016-5085(99)70065-X.

Yamaoka, Y., Kodama, T., Gutierrez, O., Kim, J. G., Kashima, K. and Graham, D. Y. (1999). Relationship between *Helicobacter pylori iceA*, *cagA*, and *vacA* status and clinical outcome: studies in four different countries. *Journal of Clinical Microbiology* **37, 2274-2279.** PMID:10364597.

Yamaoka, Y. (2010). Mechanisms of disease: *Helicobacter pylori* virulence factors. *Nature Reviews Gastroenterology and Hepatology* **7, 629-641.** DOI:10.1038/nrgastro.2010.154.

Zhou, W., Yamazaki, S., Yamakawa, A., Ohtani, M., Ito, Y., Keida, Y., Higashi, H., Hatakeyama, M., Si, J. and Azuma, T. (2004). The diversity of *vacA* and *cagA* genes of *Helicobacter pylori* in East Asia. *FEMS Immunology & Medical Microbiology* **40, 81-87.** DOI:10.1016/S0928-8244(03)00299-2.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538