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.,...
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 peptic 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 after 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 Qantaurus 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 (WG30, WiseDoc, Seoul, Korea).

Data analysis

Statistical analysis was performed by SPSS version 16. The Pearson X² 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 cagA, 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.
Table 1: Primer sequences and annealing temperatures used in this study.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (5’-3’)</th>
<th>Annealing temperature (°C)</th>
<th>PCR product (bp)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. pylori</td>
<td>CTG GAG AGA CTA AGC CCT CC</td>
<td>55</td>
<td>109</td>
<td>Ho et al., 1991</td>
</tr>
<tr>
<td>t1S rRNA</td>
<td>ATT ACT GAC GCT GAT TGT GC</td>
<td>55</td>
<td>349</td>
<td>Van Doorn et al., 1998</td>
</tr>
<tr>
<td>cagA</td>
<td>GAT AAC AGG CAA GCT TTT GAG G</td>
<td>55</td>
<td>259 (s1)</td>
<td>Van Doorn et al., 1998</td>
</tr>
<tr>
<td>vacA s1/s2</td>
<td>ATG GAA ATA CAA CAA ACA CAC</td>
<td>52</td>
<td>286 (s2)</td>
<td>Van Doorn et al., 1998</td>
</tr>
<tr>
<td>vacA s1a</td>
<td>GTG AGG AGA CTA AGC CCT AGC</td>
<td>52</td>
<td>190</td>
<td>Van Doorn et al., 1998</td>
</tr>
<tr>
<td>vacA s1b</td>
<td>GAG GCC AGG ATA CCA CAA GAG</td>
<td>55</td>
<td>187</td>
<td>Van Doorn et al., 1998</td>
</tr>
<tr>
<td>vacA s1c</td>
<td>TTA GGT TCT CCT CAA GCT TTA GGG YTT</td>
<td>55</td>
<td>259 (s1)</td>
<td>Lee et al., 2004</td>
</tr>
<tr>
<td>vacA m1/m2</td>
<td>CAA TCT TCT GCT CAA TCA AGC GAG</td>
<td>49.3</td>
<td>570 (m1)</td>
<td>Van Doorn et al., 1998</td>
</tr>
<tr>
<td>iceA1</td>
<td>GTG TTT TTA AGC GAA GTA TC</td>
<td>46</td>
<td>247</td>
<td>Van Doorn et al., 1998</td>
</tr>
<tr>
<td>iceA2</td>
<td>GGT GGG TAT ATC AGC ATT TAT</td>
<td>46°</td>
<td>229 or 334</td>
<td>Van Doorn et al., 1998</td>
</tr>
</tbody>
</table>

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 vacAs 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 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.
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*...
is more common in \textit{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 \textit{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 \textit{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, \textit{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 \textit{iceA} genotypes directly from gastric biopsies by PCR. Though we succeeded in amplifying \textit{iceA} genes only in 12/35 (34.3\%) of biopsies, our finding agrees with Secka et al. (2011) that the PCR amplification of \textit{iceA} gene directly from gastric biopsies is relatively poor. However, amongst the \textit{iceA}-positive \textit{H. pylori} infected patients in our study, \textit{iceA1} was found to be more frequently than the \textit{iceA2} (\textit{iceA1} 58.3\%, \textit{iceA2} 33.3\%) and an \textit{iceA1/iceA2} mixed genotype was observed as well. Our results showed different \textit{iceA} frequency rates when compared to a previous report (Rahman et al., 2003) which showed \textit{iceA1} and \textit{iceA2} genotypes to be almost equally distributed in Bangladeshi dyspeptic patients. However, this finding agrees with many other studies that have shown the \textit{iceA1} allele more frequently than \textit{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); \textit{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 \textit{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 \textit{cagA} and \textit{vacA} genotypes, this effect did not persist in statistical analysis.

**CONCLUSION**

In conclusion, this study for the first time in south-eastern Bangladesh identified \textit{H. pylori} virulent genotypes directly in gastric biopsies by PCR and then examined the association of these genotypes with clinical presentations. We found that \textit{cagA}, \textit{vacA} \textit{Is1} and \textit{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 \textit{H. pylori} genotypes among Bangladeshi dyspeptic patients.

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