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Prevalence of *vacA* and *cagA* Genotypes of *Helicobacter pylori* in Iranian Children with Peptic Ulcer Disease

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Abstract: The purpose of this study was to examine the presence of *H. pylori* in gastric biopsy samples from children with PUD. DNA was extracted from the biopsy samples and subjected to PCR with primers designed to detect the *glmM, vacA* and *cagA*. A total of 123 subjects were selected, 83 (67.47 %) were positive and 40 (32.53%) were negative for *H. pylori* infection. In patients with duodenal ulcer, 97% were found to be positive for *H. pylori* which was higher than that in those with gastritis (32/43, 74.4%, P=0.01). The *vacA* s1 genotype was found more often in patients with duodenal ulcer and gastric ulcer than gastritis. Duodenal ulcer patients exhibited the highest frequency of m1 allele (69.7%), followed by patients with gastric ulcer (68.75%). In contrast, the *vacA* m2 genotype was detected in 30 (93.75%) patients with gastritis. The *cagA* was detected in the mucosa specimens of 30 (90.9%) duodenal ulcer, 14 (87.5%) gastric ulcer and 17 (53.1%) gastritis. An association between *H. pylori* infection and duodenal ulcer and gastric ulcer was confirmed overall. These results show that the *vacA* s1m1/*cagA*+ genotype was predominant in Iranian children with duodenal ulcer and gastric ulcer.

Key words: *Helicobacter pylori* · Pediatric · PUD · *glmM* · *vacA* · *cagA*

INTRODUCTION

Helicobacter pylori is a spiral, gram-negative, microaerophilic bacterium that is present in the stomach of approximately half of the world's population [1]. Gastric infection by *H. pylori* is a well-recognized cause of chronic active gastritis, PUD and linked to the development of gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [2]. *H. pylori* is common in underdeveloped countries with socioeconomic status, particularly in childhood and parallels the high gastric cancer prevalence in these countries. Infection during childhood is rare in countries with a low cancer risk [3]. The age at which children are most likely to become infected is unclear, but finding in a number of cross-sectional studies suggest that infection is acquired before the age of five [4-6]. The diagnosis of *H. pylori* infection in childhood is most often made at endoscopy, for which there are many indications. Symptoms such as abdominal pain, vomiting and haematemesis may be associated with duodenal ulcer and *H. pylori* infection [7]. Studies also suggest that infection with *H. pylori* in children can increase the risk of diarrheal diseases that may lead to malnutrition and growth faltering [8, 9]. However, most colonized individual remains asymptomatic and increased disease risk is related to bacterial strain differences, epithelial responses governed by host diversity and/or specific interactions between host and microbial determinants [10, 11].

The vacuolating cytotoxin VacA [12] and the *cagA* pathogenicity island [13] are two identified virulence factors that are considered to have an important role in the pathogenesis of *H. pylori* infection. The vacuolating cytotoxin (VacA) induces the formation of intracellular vacuoles in epithelial cell lines [12]. Besides its direct cell-damaging effect *in vitro*, VacA also plays a major role in inducing cytoskeletal changes, apoptosis, suppression of epithelial proliferation and migration [14, 15]. All *H. pylori* strains carry a *vacA* gene. However, the gene is switched on in strains, which produce vacuolating toxic protein and switched off in those, which do not produce vacuolating toxic protein. The regulation of switch on and off phenomenon of *vacA* gene is poorly understood [16]. The *vacA* alleles possess one of two types of signal

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region, s1 or s2 and one of two mid-region, m1 or m2, occurring in all possible combinations [12, 17]. *H. pylori* strains with s1/m1 or s1/m2 *vacA* gene subtypes were able to produce high or moderate levels of VacA, respectively, whereas strains with s2/m2 subtype were not produce VacA [12]. The *cagA* gene (for "cytotoxin-associated gene A") product is a highly immunogenic outer membrane protein with a molecular weight of 120 to 140 kDa [18]. *H. pylori* strains with the *cag* pathogenicity island induce particularly intense inflammatory responses of the gastric epithelium [19].

H. pylori isolates are highly diverse genetically and extremely heterogeneous. Therefore, genotyping is useful in molecular epidemiological studies and identification of the predominant *H. pylori* strains that are circulating in a given geographic area. The aim of this study was to examine the presence of *H. pylori* by PCR of gastric biopsy samples from Iranian children with PUD.

MATERIALS AND METHODS

Patients and Samples: One hundred and twenty three children were subjected to endoscopy as part of the usual diagnostic protocol and two biopsies each were taken; one biopsy from each region was used to PCR. The specimens were obtained by using sterilized biopsy forceps cleansed with a detergent, disinfected with 70% ethanol and rinsed with sterile water after each examination. The biopsies were transported the same day to the laboratory in dry ice and kept at -80°C until the DNA extraction and PCR were performed. The endoscopic findings were described as gastritis, duodenal ulcer, gastric ulcer and normal mucosa. None of the patients had received antibiotics, proton pump inhibitors or bismuth during last six months. Written informed consent was obtained from the parents.

Histology: The specimens were fixed in 10% formaldehyde solution and embedded in paraffin. The pathologist was unaware of clinical and endoscopic data. Hematoxylin-eosine (H-E) staining was used for the histopathological diagnosis. Modified Giemsa stain was used for identification of *H. pylori* [20].

Preparation of *H. Pylori* Genomic DNA from Biopsy Samples: Genomic DNA was extracted with DNP™ kit (Cinnagen, Iran) according to the manufacture's instruction. The DNA concentration was measured by ultraviolet spectrophotometry at 260 nm. Isolated DNA specimens were stored at -80°C until genotyping assays were performed.

Amplification of glmM, vacA and cagA by PCR: Primers used for *H. pylori* PCR amplification were: (i) glmMF and glmMR for a 294- bp *glmM* fragment; (ii) VA1-F and VA1-R for the *vacA* s region; (iii) VA3-F,VA3-R,VA4-F and VA4-R for the *vacA* m region; (iv) cagA/Con-F and cagA/Con-R for a 402-bp *cagA* fragment. In each case, PCR was performed with 100 ng of each primer and 100 ng of template DNA as described [21]. PCR products were examined by 2% agarose gel electrophoresis. Electrophoresis was conducted at 80 V for 1 h and gels were examined under UV light.

Statistical Analysis: All data provided are expressed as mean ± standard error of the mean (SEM). The Chi-square test was used to compare the frequencies by MedCalc Software (version 9.6). A P-value of $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Patient Characteristic: One hundred and twenty three subjects were enrolled in the study. The subjects consisting of 70 boys and 53 girls were classified into 4 groups: gastritis (n= 43), gastric ulcer (n= 20), duodenal ulcer (n=34) and controls (n=26), according to the results of endoscopic and histological examination. In controls, there were no histological signs of inflammation. The demographics of the patients are listed in Table 1. Recurrent abdominal pain and chronic vomiting were the most common upper GI symptoms.

Detection of *H. pylori*: By using primers GLMF and GLMR to amplify the *glmM* gene, the expected PCR product of 294 bp was obtained in 33 (97%) patients with

Table 1: Participant characteristics

Characteristic	N (%)
Sex	70 (56.9)
Boy	53 (43)
Girl	123
total	4-14
Age (years)	
Chief complaint	
Recurrent epigastric pain	98 (79.6)
Vomiting	63 (51.2)
Heart burn	31 (25.2)
Bloating	57 (46.3)
Regurgitation	53 (43.1)
Hematemesis	28 (22.7)
Chronic diarrhea	43 (34.9)
Histology	
Gastritis	43 (34.9)
Duodenal ulcer	34 (27.6)
Gastric ulcer	20 (16.2)
Normal	26 (21.1)

Table 2: Genotype of *H. pylori* in patients with gastritis, gastric ulcer and duodenal ulcer

Groups	*cagA*	s1	s2	m1	m2
Gastritis (n=43)	17 (53.1%)	20 (62.5%)	12 (37.5%)	2 (6.25%)	30 (93.75%)
Gastric ulcer (n=20)	14 (87.5%)	13 (81.2%)	3 (18.75%)	11 (68.75%)	5 (31.25%)
Duodenal ulcer (n=34)	30 (90.9%)	29 (87.9%)	4 (12.1%)	23 (69.7%)	10 (30.3%)

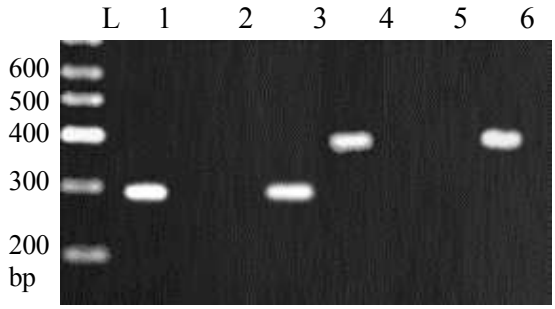

Fig. 1: Electrophoretic analysis of *glmM* and *cagA* PCR products. Lane L, DNA marker; lanes1and 3, patient's positive samples for *glmM* (294 bp); lane 2, patient's negative sample for *glmM*; lanes 4 and 6, *cagA*-positive (402 bp); lane 5, *cagA*-negative

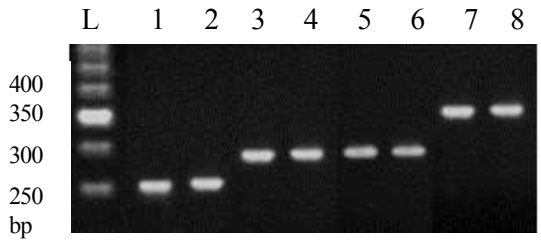

Fig. 2: Detection of *vacA* s and m alleles. Lane L, DNA marker; lanes 1 and 2, s1 allele (259 bp); lanes 3 and 4, s2 allele (286 bp); lanes 5 and 6, m1 allele (290 bp); lanes 7 and 8, m2 allele(352 bp)

duodenal ulcer (Figure 1). It was higher than that in those with gastric ulcer (16/20, 80%) and gastritis (32/43, 74.4%). We found a higher frequency of the infection in children aged 9 to 12 years. There were no differences for most of the symptom characteristics when comparing infected with non-infected children.

Determination of vacA Genotypes and cagA Status: The *vacA* gene was detectable in all *glmM*-positive biopsy speciens. Detection of vacA s1, s2, m1 and m2, alleles is shown in Figure 2. In this study, predominance of *vacA* s1 was found in patients with duodenal ulcer and gastric ulcer. In patients with gastritis, *vacA* s1 and m1 were fewer than those in patients with duodenal ulcer (62.5% vs 87.9% and 6.25% vs 69.7%, P=0.008, Table 2). The vacA s2 allele was detected in 20 (16.26%) children; 4 (12.1%) of 33 with duodenal ulcer, 3 (18.75%) of 16 with gastric ulcer, 12 (37.5%) with gastritis and 1(50%) of 2 controls. The *vacA* m1 genotype was detected in 36 (43.2%) patients, while the m2 genotype was detected in 45 (55.1%) of patients. Duodenal ulcer patients exhibited the highest frequency of m1 allele (69.7%), followed by patients with gastric ulcer (68.75%). In contrast, the *vacA* m2 genotype was detected in 30 (93.75%) patients with gastritis (Table 2). Therefore, the s1m1 type of *vacA* was commonly found in Iranian children with duodenal ulcer and gastric ulcer. Overall, 61 of the 83 infected children were *cagA* positive. Figure 1 shows that when the *cagA* gene was amplified, a 402-bp PCR product was visualized as a unique and homogenous band. *cagA* was present in 30(90.9%) children with duodenal ulcer and in 14 (87.5%) children with gastric ulcer (Table 2).

Relation Between *H. pylori* Genotype and PUD: The frequency of *vacA* s and m-alleles in the patients is shown in Table 2. 29 (87.9%) of patients with duodenal ulcer and 13 (81.2%) of patients with gastric ulcer were infected with *vacA* s1 strains compared with 20 (62.5%) from subjects with gastritis. The *vacA* m1 genotype was detected in 23 (69.7%) of 33 patients with duodenal ulcer, 11 (68.75%) of 16 with gastric ulcer and 2 (6.25%) of 32 with gastritis. The *vacA* m2 genotype was found more often in *H. pylori* from patients with gastritis 30 (93.75%) than in *H. pylori* from duodenal ulcer 10 (30.3 %, P<0.0001) or gastric ulcer 5 (31.25%, P<0.0001). A significant association was also observed between *vacA* m1 allele and *cagA* status (P<0.001), while most s2m2 samples carried a *cagA*-negative genotype.

In the present study, we examined the potential correlation between *H. pylori* virulence factors and clinical outcomes in Iranian children with PUD. In adult patients, severe gastroduodenal diseases such as chronic active gastritis, PUD, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma occur after a long-term colonization [18], while the onset of *H. pylori* infection is essentially during childhood [22]. In this study, the *glmM* gene was used for detection of *H. pylori* in biopsy samples, using PCR. We detected the *H. pylori* genomic DNA in 16 (80%) of the

20 patients with gastric ulcer, 33 (97%) of the 34 patients with duodenal ulcer. It was found that there is a significant correlation between the presence of *H. pylori* and duodenal ulcer (odds ratio= 10.31; 95%CI= 1.29-81.90; P<0.0001). The prevalence of *H. pylori* infection has been reported to be 60-90% in Iran [23; 21]. The rate of *H. pylori* infection among childhood PUD has been shown to be 33-92% in patients with duodenal ulcer and 20-70% in those with gastric ulcer [24, 25].

For the *vacA* genotype, our results showed that the *vacA* s1 allele was predominant 62(76.5%) followed by the *vacA* m2 allele 45(55.5%). Our findings demonstrate a strong association between the presence of the s1 allele and presence of a duodenal ulcer in children. A similar result was reported for adults from Iran [26, 27]. Some investigators from different parts of the world have observed an association between *H. pylori vacA* alleles and PUD, whereas some others have failed to find an association between the same. *H. pylori* genotypes in pediatric populations vary between geographic regions [28, 29]. De Gusmao *et al.* observed an association between the presence of s1 and m1 alleles of the *vacA* and the presence of duodenal ulcer in Brazilian children [30]. However, studies of Swedish and Canadian children showed that there was no association of *vacA* alleles and clinical outcome [31]. In general, s1m1 and s1m2 genotypes produce high and moderate level of toxin respectively, while s2m2 strains show little or no vacuolating toxin activity [12].

The *cagA* positive *H. pylori* strains have been known to be associated with severity of disease outcome [32]. In this study, the most significant results were that cagA+ *H. pylori* was detected in 90.9% duodenal ulcer patients and 53.1 % gastritis. In Japan [28], Korea [33] and Brazil the prevalence of the *cagA* genotype in pediatric with gastritis were, 100% 94% and 69%, respectively. However, Kato et al. found that a high prevalence of *cagA*þ positive *H. pylori* strains (80-90%) in children was not associated with gastritis or peptic ulcer disease [35].

In conclusion, this pilot study carried out in Iran focused on 97 children with duodenal ulcer, gastric ulcer and gastritis. A strong association between *H. pylori* infection and peptic ulcer was confirmed overall. These results show that the *vacA* s1m1/*cagA*+ genotype was predominant in this population of children with duodenal ulcer and gastric ulcer. Further studies with larger sample size, host genetic susceptibility and bacterial variants are needed to examine the role of *H. pylori* in children with PUD.

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