Malaysian Journal of Microbiology, Vol 7(3) 2011, pp. 167-170
http://dx.doi.org/10.21161/mjm.29010

## SHORT COMMUNICATION

# High frequency of *cagA* and *vacA* s1a/m2 Genotype among *Helicobacter pylori* Infected Gastric Biopsies of Pakistani Children

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Received 30 November 2010; received in revised form 2 February 2011; accepted 20 February 2011

**ABSTRACT**

The vacuolating cytotoxin VacA and cytotoxin associated gene product CagA, encoded by *vacA* and *cagA* are major virulence determinants associated with pathogenesis of *Helicobacter pylori*. The presence and prevalence of two major *H. pylori* virulence associated genes among gastric biopsies of Pakistani children were investigated in the current study. Fifty one gastric biopsy specimens of children were analysed for 16S rRNA, *vacA* and *cagA* genes using PCR. The results showed that 21 (41.2%) biopsies were positive for *H. pylori* as determined by 16S rRNA PCR. In the 21 *H. pylori* positive gastric biopsies, 19 (90.5%) showed *vacA* s1a, 1 (4.75%) was *vacA* s1b and 1 (4.75%) was *vacA* s2 whereas, 5 (23.8%) were *vacA* m1 and 16 (76.2%) were *vacA* m2. None of the *H. pylori* positive biopsies carried *vacA* s1c subtype. The *cagA* gene was found in 13 (61.9%) of *H. pylori* infected biopsies and different *vacA* combinations were found with or without *cagA* gene. *H. pylori* was detected with high frequency of *cagA* while *vacA* s1a and *vacA* m2 regions with *vacA* s1a/m2 genotype were predominant in *H. pylori* infected gastric biopsies of children.

*Keywords: H. pylori*, Frequency, Children, *vacA*, *cagA*, PCR

## INTRODUCTION

*Helicobacter pylori* is the major infectious agent of chronic superficial gastritis and plays a central role in the development of peptic ulcer disease and adenocarcinoma (Al-Shamahy, 2005), hence classified as a class I carcinogen by the World Health Organization (International Agency for Research on Cancer, 1994). The outcome of infection depends on the genetic makeup of the host, the pathogen and the environment. Many virulent factors have been identified which enable *H. pylori* in invading, surviving and colonizing the hostile acidic environment of the human stomach. The two most important factors that determine the outcome of infection include *vacA* and *cagA* genes. Evidence suggests that *H. pylori* infection is a risk for development of gastric carcinoma whereas, *vacA* and *cagA* genes can be used as indicators of its virulence level (Cittelly *et al*., 2002).

DNA analysis has revealed that *vacA* has a mosaic structure comprising allelic variations in the signal and mid region, each having two different alleles (s1a, s1b, s1c/s2 and m1/m2) with different biological activities (Atherton *et al*., 1995; Ahmad *et al*., 2009). In general, *vacA* type s1/m1 and s1/m2 strains produce high and moderate levels of toxin, whereas s2/m2 strains produce little or no toxin (van Doorn *et al*., 1998).

There are reports that *cagA* is present in more than 50% of the *H. pylori* strains and it is a marker for the presence of cag-pathogenicity island and is associated with peptic ulcer and gastric malignancy (Kidd *et al*., 2001). The basic purpose of the current study was to determine the status of *vacA* and *cagA* genes in *H. pylori* infected gastric biopsies of Pakistani children.

## MATERIALS AND METHODS

### Subjects

This was a prospective study and all children appearing at KRL General Hospital, Islamabad and Military Hospital, Rawalpindi, Pakistan with symptoms suggestive of upper gastrointestinal endoscopy were enrolled. The protocols were approved by The Research Ethics Committee of Pakistan Institute of Nuclear Science and Technology (PINSTECH) and written informed consent was obtained from parents/guardians of the subjects. Gastric biopsies collected from 51 children (age range: 3 to 18 years) were studied to determine the presence of *H. pylori* and to test the distribution of its two major virulence determinants, *vacA* and *cagA*.

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

**DNA Extraction**

DNA was extracted by using ethanol precipitation method from gastric biopsies of patients. Gastric biopsies were crushed manually in an eppendorf tube. The crushed material was suspended in a medium consisting of 20 µL of 10% SDS, 80 µL of proteinase K buffer (0.5 M EDTA and 4 M NaCl, pH 7.5) and 40 µL of proteinase K (10 mg/mL). The mixture was incubated at 55 °C for 5 h, then 100 µL of 6 M NaCl was added and vortexed for 1 min followed by centrifugation at 14,000 rpm for 1 min. The nucleic acids were precipitated by adding chilled absolute ethanol and harvested by centrifugation. After centrifugation DNA pellet was washed with 70% ethanol and resuspended in 100 µL of TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5). Samples were stored at –4 °C till further use.

**PCR**

All PCR reagents were supplied by Promega Corporation (Madison, USA) and primers by Alpha DNA (Montreal, Canada) and Gene Link (Hawthorne, NY, USA). Each PCR reaction mixture consisted of 1× green reaction buffer, 1.5 mM $MgCl_2$, 200 µM each dNTP, 10 pmol of each primer, 1.5 U Taq DNA polymerase and 5 µL of target DNA (final reaction volume of 25 µL). Target gene, amplicon size, primer names and sequences are shown in Table 1. PCR amplification was performed under the following conditions: initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing (see Table 1 for annealing temperatures specific to each primer pair used) for 30 sec, polymerization at 72 °C for 30 s and final polymerization at 72 °C for 5 min. PCR was performed on T Professional Basic Thermocycler and DNA templates of *H. pylori* strains G27 and SS1 were used as positive controls. PCR mixture was subjected to gel electrophoresis (1.5% agarose) with 100 bp DNA ladder and ethidium bromide staining for detection of amplified products.

**Statistical Analysis**

The chi-square test was used for analysis of categorical data. Significance was defined as *p* value of less than 0.05.

**RESULTS**

The study subjects consisted of 51 children [29 (56.9%) males and 22 (43.1%) females] with age range of 3 to 18 years. Based on 16S rRNA PCR, 21 (41.2%) gastric biopsies were positive for *H. pylori* DNA. Out of the 21 subjects positive for *H. pylori*, 14 were male (48.3%) and 7 were female (31.8%).

The polymorphism in signal sequence and middle region of *vacA* was typed by PCR in all *H*. *pylori* positive gastric biopsies. Among *H*. *pylori* positive gastric biopsies, 19 (90.5%) were positive for *vacA* s1a, 1 (4.75%) for *vacA* s1b and 1 (4.75%) for *vacA* s2 whereas, 5 (23.8%) were *vacA* m1 and 16 (76.2%) were *vacA* m2 positive. All of these positive *H. pylori* biopsies were negative for *vacA* s1c subtype.

Of the 21 gastric biopsy specimens that showed positive amplification in 16S rRNA gene, 13 (61.9%) were positive for *cagA* gene. All negative biopsies for 16S rRNA gene were also negative for *cagA* and *vacA* genes. *vacA* s1a/m1 and s1a/m2 genotypes with *cagA*+ were found in 4 (30.8%) and 8 (69.2%) respectively but the correlation was not significant ($p = 0.516$), while the *vacA* s1a/m1, s1a/m2 genotypes with *cagA*- were detected in 1 (12.5%) and 6 (62.5%) of the 21 cases, respectively. *vacA* genotypes s1b/m2 and s2/m2 were found in two *cagA*- strains whereas these could not be seen in *cagA*+ strains.

**Table 1:** Primer details of 16S rRNA, *vacA* and *cagA* genes

| Target site | Amplicon size (bp) | Primer names and sequences | Annealing temperature | References |
|---|---|---|---|---|
| 16S rRNA | 138 | HPF (5' GCGACCTGCTGGAACATTAC 3')<br>HPR (5' CGTTAGCTGCATTACTGGAGA 3') | 60 °C | GramLey, *et al.,* 1999 |
| *vacA* s1a | 190 | AA1F (5' GTCAGCATCACACCGCAAC 3')<br>AA1R (5' CTGCTTGAATGCGCCAAAC 3') | 56 °C | Atherton *et al*., 1995 |
| *vacA* s1b | 187 | SS3F (5' AGCGCCATACCGCAAGAG 3')<br>SS3R (5' CTGCTTGAATGCGCCAAAC 3') | 56 °C | Atherton *et al*., 1995 |
| *vacA* s1c | 213 | S1CF (5' CTCGCTTTAGTGGGGCTA 3')<br>S1CR (5' CTGCTTGAATGCGCCAAAC 3') | 56 °C | Yamaoka *et al*., 1999 |
| *vacA* s2 | 199 | SS2F (5' GCTAACACGCCAAATGATCC 3')<br>SS2R (5' CTGCTTGAATGCGCCAAAC 3') | 56 °C | Atherton *et al*., 1995 |
| *vacA* m1/m2 | 570/645 | VAGF (5'CAATCTGTCCAATC AAGCGAG 3')<br>VAGR (5'GCGTCTAAATAATTCCAAGG 3') | 57 °C | Yamaoka *et al*., 1999 |
| *cagA* | 189 | CAGAF (5' TTGACCAACAACCACAAACCGAAG 3')<br>CAGAR (5' CTTCCCTTAATTGCGAGATTCC 3') | 62 °C | van Doorn *et al*., 1998 |

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

## DISCUSSION

There is geographical variation in distribution of *H. pylori* genotypes and very little information about frequency of *H. pylori vacA* genotypes and *cagA* in Pakistan is available. In the present study, we determined the *vacA* and *cagA* status among *H. pylori* infected gastric biopsies of children.

All the strains of *H. pylori* have *vacA* gene but its structure varies, mainly in the region encoding the signal sequence (type s1a, s1b, s1c or s2) and the mid-region (type m1 or m2), (Atherton *et al*., 1995). A strain's *vacA* structure determines its *in-vitro* cytotoxin activity, with *vacA* type m1 being more active than type m2, type s1a being more active than type s1b and type s2 *vacA* not producing detectable activity. Recently, Ahmad *et al.,* (2009) found *vacA* s1b and *vacA* m2 as main subtypes in gastric biopsies of adult patients from Rawalpindi while *vacA* s1a and *vacA* m1 as predominant subtypes were reported mainly in adult patients of Karachi, Pakistan (Yakoob *et al*., 2009). Our results showed that the predominant *vacA* genes were s1a and m2 in *H. pylori* infected gastric biopsies of children which were similar to the report of Hou *et al*., (2000) who observed that s1a and m2 were more prevalent *vacA* genes in China while Mishra *et al*., (2002) in India found *vacA* s1a and *vacA* m1 were major subtypes in gastric biopsies of patients referred for endoscopy.

More prevalent *vacA* genotype in a recent report from Pakistan was s1b/m2 (54.5%) in adult dyspeptic patients (Ahmad *et al*., 2009), while in India predominant *vacA* genotype was s1a/m1 (53.2%), (Mishra *et al*., 2002). This is in contrast to current study where *vacA* genotype s1a/m2 was detected in 66.7% of *H. pylori* infected gastric biopsies of children, which was similar to the same *vacA* genotype reported in China with the frequency of 90.5% (Hou *et al*., 2000). The *vacA* genotype s1b/m1, s1c/m1, s1c/m2 or s2/m1 was not detected in this study.

Ahmad, *et al*., (2009) described a 24.2% prevalence of *cagA* gene in gastric biopsies obtained from adult dyspeptic patients from Rawalpindi area and Yakoob *et al*., (2009) found 56% frequency of *cagA* in adult patients from Karachi, while our data involved gastric biopsy specimens collected from children and reported a higher prevalence of *cagA* viz. 61.9%. This frequency of *cagA* is low as compared to our neighboring countries where the frequency of *cagA* was 93.2% in China (Hou *et al*., 2000), 76% in Iran (Jafari *et al*., 2008) and 96.2% in India (Mishra *et al*., 2002).

In the study presented here, we have reported high frequency of *cagA* (61.9%) with predominant *vacA* s1a/m2 genotype in *H. pylori* infected gastric biopsies of Pakistani children, which to our knowledge has not been previously published. We investigated frequency of *cagA* and *vacA* genotype among Pakistani children which was different from the information provided by authors of same region who reported the frequencies in adults. Our investigation set here a question: Is there a different genetic makeup of *H. pylori* strains infecting children and adults of this region? Nevertheless, this study provides a basis for future detailed investigations to understand genetic diversity of *H. pylori* among Pakistani population.

## ACKNOWLEDGEMENT

This study was financially supported by Pakistan Science Foundation through project no. PSF/res/C-PINSTECH/Med(207). Thanks to Musharaff Jellani for his technical assistance during this study and we are also thankful to Muhammad Afzal Javed for his comments and suggestions in writing of this paper.

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

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