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Original Article

Microbiology Section

Prevalence of Specific *Helicobacter Pylori cagA, vacA, iceA, ureC* Genotypes and its Clinical Relevance in the Patients with Acid-Peptic Diseases

HIMANI BHARDWAJ PANDYA[1], HARIHAR HARDAS AGRAVAT[2], JAGDISH SHANTILAL PATEL[3]

ABSTRACT

Introduction: Virulent markers of *H. pylori*, the vacuolating cytotoxin (*vacA*), cytotoxin-associated gene A (*cagA*), induced by contact with epithelium factor antigen (*iceA* gene) and the urease C gene (*ureC*) may plays a major role in determining the clinical outcome of *Helicobacter* infections.

Aim: To detect the prevalence of the *cagA, vacA, ureC* and *iceA* genotypes of *H. pylori* from antral biopsy specimens of patients and to associate its role in specific disease.

Materials and Methods: The study was conducted at Department of Microbiology of Shree P.M. Patel College of Paramedical Sciences, Anand, Gujarat, India. Seventy one antral biopsies of symptomatic patients referred for endoscopy from October 2012 to September 2013 were subjected to Multiplex PCR. DNA isolation from 71 biopsy samples was done by using "QIAamp DNA mini kit" from QIAGEN (GmbH, Hilden, Germany). Data was analysed using Chi square (χ^2) test and p-value<0.05 was considered significant.

Results: Out of the 71 biopsies screened, 22(31%) samples were positive for *H. pylori* by PCR, with high proportion of *cagA* positive (17/22 specimen; 77.27%), followed by *ureC* positive (4/22 specimen; 18.18%) and *vacA* positive (1/22 specimen; 4.54%) strains. Significant association was found between *cagA* and female gender (p-value=0.042). Out of 17 *cagA* positive strains, 9(52.94%) were found in patients with gastritis, 5(29.41%) in reflux oesophagitis and 3(17.64%) in patients with diodenal ulcer. We found 0% prevalence of *iceA* gene; conversely we had three peptic ulcer patients with only *cagA* positivity.

Conclusion: The *cagA* positive strain mainly affects the patients with gastritis specifically of female gender and *iceA* genotype is not a useful marker associated with peptic ulcer disease. Patients should be screened for *cagA* genotype when reported to be a case of gastritis for early treatment to prevent further complications such as cancer.

Keywords: Cytotoxin associated gene A, Gastritis, Genotyping, *Helicobacter pylori*

INTRODUCTION

Ever since its discovery in 1982 by Warren JR and Marshall B [1], the organism has generated tremendous interest among the medical fraternity. *H. pylori* are major etiological agent for the development of chronic gastritis, gastroduodenal ulcers and gastric adenocarcinoma [2]. Genotyping is very useful in molecular epidemiological studies and identification of predominant strains as *H. pylori* isolates tend to be diverse genetically with heterogeneous distribution [3]. The *cag* and *vac* markers tend to play a major role in determining clinical outcome [4]. The above virulence factors are found in a subset of clinical isolates such as *cagA, vacA*, *iceA*, *ureC* [4]. The *cagA* is frequently associated with cytotoxin production and the induction of cytokines like Interleukin 8 (IL8) by gastric epithelial cells [5]. Studies have suggested that *cagA* is a useful marker for the most virulent strains that are associated with peptic ulcer, atrophic gastritis and adenocarcinoma and presenting about 60% to 70% of *H. pylori* strains [5,6]. The *vacA* is also one of the major virulence factors, which is encoded by the *vacA* gene and this 87 kDa protein induces both vacuole formation and apoptosis in gastric epithelium [7,8]. The heterogenecity exist at the middle (m) and the signal (s) region of *vacA*, due to which there is a considerable variation in the vacuolating activity in strains [8]. The *iceA* gene (a gene induced by contact with gastric epithelium) produces high levels of cytokine IL-8 in gastric mucosa and hold higher rates of peptic ulcer disease [9]. Molecular methods like polymerase chain reaction have the potential to accurately determine both the presence of infection and the genotype of bacteria; and looks very permissive as well as sensitive and specific test for the detection of *H. pylori* genotypes [9].

To predict the clinical outcomes of the infection and also for better understanding the distribution of microorganism and its evolutionary origins, it is very important to study the diversities of *H. pylori* genes. In the present study, we tried to explore the distribution of various genotypes of *H. pylori* isolates from biopsy specimens and also to establish the potential association with the clinical outcome.

MATERIALS AND METHODS

This prospective cross-sectional study was conducted at the Department of Microbiology, Shree P.M. Patel college of Paramedical Science and Technology, Anand, Gujarat, for a period of one year from October 2012 to September 2013.

Design of the Study

Seventy one consecutive (46 males and 25 females, age; 10-90 years), symptomatic patients attending the endoscopic unit of "deep surgical hospital" were included in this study. Based on endoscopic findings, out of 71 patients, 34 patients were suffering from gastritis, 26 with reflux oesophagitis, nine with duodenal ulcer and two with duodenitis. Patients taking aspirin or Non-Steroidal Anti-Inflammatory Drugs (NSAIDS) in the past four week or those on Proton Pump Inhibitors (PPI) or patients with previous therapy to eradicate *H. pylori*, or if the inform consent was not obtained were excluded from the study.

Sample

One antral biopsy was collected from each symptomatic patient in fasting condition using Olympus Video Endoscope 1306.

Ethical Considerations

The present study got ethical approval from Human Research Ethics Committee (HREC) of H.M. Patel Center for Medical Care and Education, Pramukh Swami Medical College, Karamsad, Gujarat, India.

DNA Extraction from Biopsy Sample

DNA isolation from 71 biopsy specimens was done by using "QIAamp DNA mini kit" (Qiagen GmbH, Hilden, Germany, Lot No: 11872534, Cat No: 51306) as described by the manufacturer.

Genomic DNA Isolation

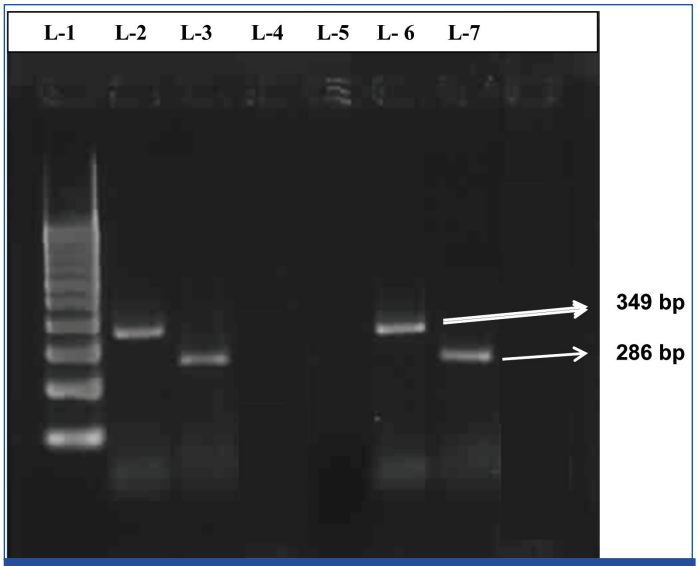
The tissue biopsies were centrifuged at 5000 rpm for 10 min and re-suspended in 200 µL of ATL (Animal Tissue Lysis) buffer for complete lysis. Finally, the DNA was eluted in 100 µL of elution buffer. DNA purity and quantity was determined using a GeneSys 10UV spectrophotometer (Thermo Scientific, USA). The extracted DNA was stored at -20ºC until used as template in PCR.

H. pylori Genotyping

PCR reaction was carried out in a final volume of 50 µL as follows. 5 µL of 1X PCR buffer (50 mM KCL, 10 mM tris HCL, 2.5 mM $MgCl_2$, 0.01% gelatin), 8 µL of 0.2 mM dNTPs, 0.5 µL each primer (Procured from Bangalore Genie), 0.6 µL of Taq DNA polymerase, 6 µL DNA sample and 25.4 µL distilled water. Thermal cycler (ESCO-2720) of Applied Biosystem was used for amplification. [Table/Fig-1] summarizes the primer sequences, expected size of the product and PCR conditions. The amplified PCR products were analysed by agarose gel electrophoresis. An 8µL of PCR product were run on a 2.5% agarose gel, containing ethidium bromide and were visualized under ultraviolet light source.

Gene	Primer sequence	PCR conditions	Size of the PCR product (bp)	Reference
cagA	F-5′-GATAACAGCC AAGCTTTTGAGG-3′ R-3′-CTGCAAAAGA TTGTTTGGCAGA-5′	denaturation for 1 min at 94°C; annealing for 1 min at 56°C and extension for 1 min at 72°C (35 cycles)	349	[15]
ureC	F-5′-AAGCTTTTAGG GGTGTTAGGGGTT-3′ R-3′-AAGCTTACTTT CTAACACTAACGC-5′	denaturation for 1 minute at 93 °C; annealing for 1 minute at 55 °C and extension for 1 minute at 72 °C (35 cycles)	294	[15]
vacA	F-5′-ATGGAAATA CAACAAACACAC-3′ R- 3′-CTGCTTGA ATGCGCCAAAC-5′	denaturation for 1 min at 94°C; annealing for 1 min at 53°C and extension for 1 min at 72°C (35 cycles)	286	[15]
iceA	F-5′-GTGTTTTTAA CCAAAGTATC-3′ R-3′-CTATAGCCA TTATCTTTGCA-5′	denaturation for 1 min at 94°C; annealing for 1 min at 56°C and extension for 1 min at 72°C (35 cycles)	247	[15]

[Table/Fig-1]: PCR primers and conditions.
F: Forward primer; R: Reverse primer; bp: Base pairs



[Table/Fig-2]: Separation of PCR products by 2.5% agarose gel electrophoresis.
Legend for figure: Lane 1: 100bp DNA ladder, Lane 2 and 3: *cagA* positive control and *vacA* positive control, lane 6: *cagA* gene (349bp), Lane 7: *vacA* gene (286bp), Lane 4: negative control.

STATISTICAL ANALYSIS

The Fischer's-exact and Chi square (χ^2) test were used to compare the relationship between *H. pylori* genotypes and clinical outcome. A p-value of < 0.05 was considered statistically significant.

RESULTS

Detection of the Three Genes by PCR

Out of the 71 biopsies screened, 22 (31%) samples were positive for *H. pylori* by PCR, with high proportion of *cagA* positive (17/22 specimen; 77.27%), followed by *ureC* positive (4/ 22 specimen; 18.18%) and *vacA* positive (1/22 specimen; 4.54%) strains [Table/Fig-2]. we did not find any strain with *iceA* gene in our province. Forty nine (69%) biopsy samples were negative for all the four genes screened.

Association of cagA gene with various demographic factors like age group and gender as well as with gastrointestinal diseases

Prevalence of *cagA* was found higher in age group 21-40 years. (52.94%), followed by 41-60 years (35.29%) and 61-80 years. (11.76%). High incidence of *cagA* positive *H. pylori* strains found in adults, may suggest that this population may be at risk for developing more serious pathology of the gastric mucosa. We found significant association between *cagA* and gender of the patient; prevalence was higher in females (10/17, 58.8%), as compared to males (7/17, 41.2%) (p-value 0.042 < 0.05) [Table/Fig-3]. We found that out of 17 *cagA* positive strains, 9(52.94%) were found in patients with gastritis, 5(29.41%) in reflux oesophagitis and 3(17.64%) in patients with diodenal ulcer [Table/Fig-4].

Detection of ureC gene and vacA and its correlation with various demographic factors like age group, gender and Gastro-intestinal disease

The *ureC* gene (four strains positive) was found to be equally distributed (50%) in both the gender and age-group of 21-40 and 41-60 years. Prevalence of *ureC* genotype was 50% in gastritis patient as well as in reflux oesophagitis patients [Table/Fig-4]. We found only one strain positive for *vacA* in a male patients of reflux oesophagitis belonging to age group of 61-80 years.

Genes	Male	Female	chi-square (p-value)
cag A	7	10	4.10 (0.042)*
vac A	1	0	N.A.
ice A	0	0	N.A.
ure C	2	2	0.258(0.612)

[Table/Fig-3]: Distribution of *H. pylori* genotypes in different gender.
Chi square (χ^2) test, *p-value < 0.05 is significant.

DISCUSSION

H. pylori possess a remarkable degree of genetic diversity, which is closely related with its epidemiological and pathological characteristics, as a result genotyping becomes very important to characterize the strains and even if many infections are clinically silent, but the patient infected with *H. pylori* presents increased morbidity and mortality [10,11]. The present study was designed

Genotype		Duodenal Ulcer (n=9)	Duodenitis (n=2)	Gastritis (n=34)	Reflux oesophagitis (n=26)
cagA (17+ve)	Negative	6	2	25	21
	Positive	3	0	9	5
	Chi-square (p-value)	0.499 (0.480)	0.648 (0.421)	0.229 (0.632)	0.500 (0.479)
vacA (1+ve)	Negative	9	2	34	25
	Positive	0	0	0	1
	Chi-square (p-value)	0.147 (0.701)	0.029 (0.864)	0.932 (0.334)	1.755 (0.185)
ureC (4+ve)	Negative	9	2	32	24
	Positive	0	0	2	2
	Chi-square (p-value)	0.615 (0.433)	0.123 (0.726)	0.008 (0.931)	0.327 (0.567)

[Table/Fig-4]: Prevalence of *H. pylori cagA, vacA*, *ure C* genotypes in various endoscopic findings.

to analyse the genetic distribution of various genes of *H. pylori* from biopsy samples and to associate its role in the clinical outcome of infections. We found that prevalence of *cagA* was 77.27% (17/22), which was in conformity to various studies done where the prevalence varies from 62%-77% respectively [12,13], while in one study done by Udhayakumar G et al., prevalence of *cagA* positive strain was 96% [14]. The prevalence of *cagA* genotypes in Anand district is relatively important to reveal the circulating *H. pylori* strains in a given geographic region and the associated need for region-specific diagnostics for *H. pylori* virulence markers. The present study revealed a relatively high prevalence of the more virulent allele *cagA* suggesting a common prevalence of virulent *H. pylori* genotypes. The *cagA* positive *H. pylori* was detected in 52.94% of gastritis patients (p-value, 0.632) and 17.64% of duodenal ulcer patients (p-value, 0.480), statistically the correlation is not found significant, but 9 of 22 *cagA* positive strains shows emergence of this promising gene in Anand district in relation with the patients suffering from acute gastritis. Results are quite in accordance with many studies done, which proves its predominance in causing gastritis [15,16]. We also found significant association between *cagA* and female gender (p-value, 0.042), which is in contrast to the study done by Abadi ATB et al., who proved that the presence or absence of the *cagA* gene is not significantly linked to the gender [17].

H. pylori and Gastroesophageal Reflux Disease (GERD): An Unsolved Mystery

Studies have found a negative association between the presence of positive *cagA* and gastroesophageal reflux diseases [18]. The relationship between *cagA* positive *H. pylori* and complications of GERD is still controversial and the role of virulence markers of the bacterium has not been evaluated in most studies of GERD. In our study, we have attended 26 reflux oesophagitis patients, out of which eight patients were positive for *H. pylori* infection and out of which, 5 (19.23%) were *cagA* positive strains, 2 (7.69%) were *ureC* strains and 1 (3.84%) *vacA* positive strain [Table/Fig-4]. From the above results, we cannot remark on any protective association between them as majority of *H. pylori* infected GERD patients were having *cagA* genotype.

Detection of iceA Gene in Gastric Specimen and in their Corresponding *H. pylori* Isolates

In our study, we have 0% prevalence of this gene, conversely we had three peptic ulcer patients and all the three were *cagA* positive but *iceA* negative. In contrast to our results, a study done by Peek RM et al., showed an association between the allelic variant *iceA1* and more severe inflammation of gastric mucosa [19]. Similar studies showing no association between *iceA* gene and peptic ulcer have been reported from Turkey and Lithuania [20,21]. We were unable to confirm an association between the *iceA* gene and clinical outcome. This finding may reflect important geographic differences between *H. pylori* and patients. As it is a well known fact that *H. pylori* genotypes are not uniformly distributed all over the world.

To the best of our information, this is the first study done in Gujarat state of Western India to find the clinical relevance of putative virulence-associated genes of *H. pylori* in patients with gastric diseases.

LIMITATION

The most important constraint of the study is the sample size; large studies with more number of samples are required to prove the role of these markers in virulence.

CONCLUSION

The virulence strains with *cagA* genotype extend at our province, may result in severe clinical outcomes such as ulcers which may be developed to cancer. We also draw focus on the 0% prevalence of *iceA* gene in peptic ulcer patients, which concludes that *iceA* genotype is not a useful marker of virulence in this population and that the progression from gastritis to peptic ulcer must require some other genes or factors including the genetic susceptibility of the host.

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PARTICULARS OF CONTRIBUTORS:
1. Tutor, Department of Microbiology, SBKS Medical Institute and Research Center, Vadodara, Gujarat, India.
2. Dean, Department of Microbiology, Gujarat Adani Institute of Medical Sciences, Bhuj, Gujarat, India.
3. Head, Department of Biochemistry, P. D. Patel Institute of Applied Sciences, Charotar University of Science and Technology (CHARUSAT), Changa, Gujarat, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Himani Bhardwaj Pandya,
Tutor, Department of Microbiology, SBKS Medical Institute and Research Center, Piparia,
Waghodia, Vadodara-391760, Gujarat, India.
E-mail: himani22pandya@yahoo.com

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