

Special Article - Digestive Endoscopy

Determination of *Helicobacter pylori* Virulence Genes in Clinical Isolates of Symptomatic Patients from South Coastal Region of Karnataka – A Preliminary Work

Shetty V¹, Ballal M^{1*}, Lingadakai R² and Mukhopadhyay AK³

¹Department of Microbiology, Manipal University, India

²Department of Surgery, Manipal University, India

³Bacteriology Division, National Institute of Cholera and Enteric Diseases, Kolkata

*Corresponding author: Ballal M, Department of Microbiology, Enteric Pathogens Division, Manipal University, Manipal, Karnataka State, Pin: 576104, India, Tel: +91 0820-2933556; Emails: mamatha_98@yahoo.com

Received: November 28, 2014; Accepted: January 28, 2015; Published: January 30, 2015

Abstract

Introduction: *Helicobacter pylori* is a microaerophilic, spiral shaped, unipolar flagellated, gram negative organism which has high tropism towards gastric epithelial cells and may lead to a severe form of gastroduodenal disease. Prevalence of *H. pylori* infections in developing countries may exceed 70% but only a fraction of people develop severe disease. The involvement of *H. pylori* virulence factors *cagA* and *vacA* genotypes are well studied and show high genetic diversity geographically. The present study was conducted to detect the Presence of *H. pylori* in biopsy samples from symptomatic patients by Rapid Urease Test, histopathological examination and Culture. It also involved the detection of virulence genes *cagA*, *vacA* (s1m1, s1m2 and s2m2) genotypes from the isolated strains.

Materials and Methods: Patients with complaints of abdominal pain, discomfort, acidity and loss of appetite were chosen for endoscopy. A detailed history was taken and physical examination of the patients was carried out prior to endoscopy. A total of 3 antral biopsies were obtained from each patient and subjected to RUT, HPE and culture. Isolated *H. pylori* were further examined for the virulence genes by using PCR.

Results: Antral biopsies were collected from a total of 38 patients. Identification of *H. pylori* was performed on all gastric biopsies by RUT, HPE and Culture. The rate of positive *H. pylori* in the biopsies tested with RUT were (36.9%), HPE (42.1%) and Culture (39.5%) and the presence of virulence gene *cagA* (93.3%) and *vacA* alleles- s1m1 (60%), s1m2 (40%). None of them showed presence of *vacA* s2m2 genotypes by PCR.

Conclusion: This study provides important information regarding the rate of identification of *Helicobacter pylori* from RUT, HPE, Culture and also rate of virulence genes harbored by strains isolated from this part of Karnataka., India.

Keywords: *H. pylori*; *cagA* gene; *vacA* gene; PCR; Biopsy

Introduction

Helicobacter pylori is a slow-growing, spiral shaped, 2 to 6 unipolar flagellated, gram negative microaerophilic organism which inhabits the human stomach and has a high tropism for the gastric epithelium causing long term colonization of the gastric mucosa. It has been linked to primary cause of upper gastrointestinal disorders, such as chronic active gastritis, peptic ulcer, gastric cancer and MALT lymphoma [1-3]. Most importantly it is a significant risk factor for developing gastric cancer and WHO has designated this bacterium as class 1 carcinogen [4]. The prevalence of *H. pylori* infection varies geographically, and it may exceed 70% in developing countries. The prevalence of *H. pylori* infection is high (49.94%-83.30%) in India, interestingly, only fraction of people get infection in their lifetime [5-8].

There are several factors which are involved in severity of infection like environmental, host genetic and bacterial virulence factors [9]. The involvement of *H. pylori* virulence factors like cytotoxin associated gene (*cagA*) and vacuolating cytotoxin gene (*vacA*) genotypes are well

studied and showed the high genetic diversity geographically. The *cagA* gene, a marker for the *cag* Pathogenicity Island (*cagPAI*) which includes a number of other genes associated with increased virulence, is associated with severe clinical disease and *vacA* gene encodes for the vacuolating cytotoxin, the pore forming toxin which leads to gastric epithelial cell injury. The vacuolating activity in host cells varies due to mosaicism of the *vacA* gene in signal (s) and median (m) regions [10-12].

The aim of the present study was to detect *H. pylori* from gastric biopsy of symptomatic patients and comparison of different methods used for their detection (RUT, HPE, and Culture) and also to determine the rate of virulence genes, *cagA* and *vacA* and their alleles by PCR in this part of south coastal region of Karnataka, India.

Methods and Materials

Specimen collection and Processing

A total of 38 adult patients of both genders having complaints of abdominal pain, discomfort, acidity and loss of appetite were

Table 1: Primer used for amplification of *H. pylori* genes.

Primers	(5'-3') Sequence	(bp) Size	Anneling temp. °C
<i>cagA5cf</i> <i>cagA3cR</i>	GTTGATAACGCTGTCGCTTCA GGGTTGTATGATATTTCCATAA	350	55
<i>Va1F</i> <i>Va1R</i>	ATGGAAATACAACAAACACAC CTGCTTGAATGCGCAAAC	259(S1) 287(S2)	55
<i>vagF</i> <i>vagR</i>	CAATCTGTCCAATCAAGCGAG GCGTCAAATAATCCAAGG	550(m1) 600(m2)	55

chosen for endoscopy and detailed history was taken and a physical examination was carried out prior to endoscopy. Patients' consent to participate in the study was obtained as per the protocol of institutional ethical committee. A total of 3 Antral biopsies were obtained from each patient and one subjected to rapid urease test, one in 10% formalin processed for histopathological examination and one in 0.6ml brucella broth with 15% glycerol as transport media stored at -70° C until cultured.

Histopathological examination

Tissue biopsies treated with formalin was stained routinely with Haematoxylin and Eosin, special stains such as Giemsa was used as and when required.

H. pylori Culture

Transport medium containing the biopsy samples were vortexed, and later 100 µl plated on BHIA supplemented with, 0.4% Isovitalax, 7% horse serum, and *H. pylori* dent supplement. Cultured plates then incubated in a microaerobic atmosphere (5% O₂, 10% CO₂, and 85% N₂) at 37°C for a period of 3 to 7 days. *H. pylori* identified based on their typical morphology, gram staining, and positive reaction for urease, oxidase and catalase test.

Extraction of genomic DNA

Cells were harvested from culture plates and washed with PBS (pH8.0). The CTAB (hexadecyltrimethyl ammonium bromide) method was used for extracting the DNA.

PCR amplification

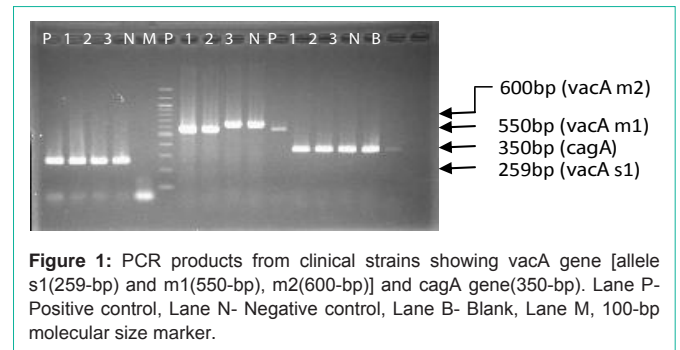
PCR amplification was done in final volume of 20µl which contained 2µl template DNA, 2µl dNTP, 1.2µl MgCl₂, 2µl 10x buffer, and 10 pmol of the appropriate primers (Table 1) in the presence of 1U Taq DNA Polymerase. The cycling condition were: Initial denaturation at 95° C for 3 min, followed by 30 cycles of denaturation at 94° C for 30sec, annealing at 55° C for 30 sec and extension at 72° C for 1 min, with final extension at 72° C for 7 min.

Results

A total of 38 dyspeptic patients, 25 males and 13 Females ranging in age from 22 to 72 years (average age of 49.6 + 13.3) were included in the study. Antral biopsies were collected from 38 patients. Identification of *H. pylori* was performed on all gastric biopsies by Rapid Urease test, Histopathology and Culture. The rate of positive *H. pylori* in the biopsies tested with RUT were (36.9%), HPE (42.1%) and Culture (39.5%) (Table 2). Histopathology of the samples positive for

Table 2: Rate of detection of *H. pylori*.

	RUT	HPE	Culture
Positive	14(36.9%)	16(42.1%)	15(39.5%)
Negative	24(63.1%)	22(57.9%)	23(60.5%)
Total	38(100%)	38(100%)	38(100%)

**Figure 1:** PCR products from clinical strains showing *vacA* gene [allele s1(259-bp) and m1(550-bp), m2(600-bp)] and *cagA* gene(350-bp). Lane P- Positive control, Lane N- Negative control, Lane B- Blank, Lane M, 100-bp molecular size marker.

H. pylori revealed, mild and chronic active gastritis (n=11), follicular gastritis (n=3) and chronic gastritis with Intestinal metaplasia (n=2).

The distribution of *cagA* gene and *vacA* gene (found in different allelic forms depending on the genotypes of the signal region and middle region) among 15 isolated strains was determined using specific primers (Figure 1) and the presence of virulence gene *cagA* (93.3%) and *vacA* alleles- s1m1 (60%), s1m2 (40%), no *vacA* s2m2 genotype from isolated *H. pylori* strains were determined by PCR. Virulence genes combinations show that 8 *H. pylori* strains had the *vacAs1m1/cagA+* combination, while 1 were *vacAs1m1/cagA-* and 6 were *vacAs1m2/cagA+* genotypes observed (Table 3).

Discussion

A reliable primary diagnosis of *H. pylori* infection is crucial in management of infection for patients with different patterns of disease. This study was conducted to compare the results of rate of identification of *H. pylori* infection from invasive methods by three different tests like Rapid Urease Test (RUT), Histopathological Examination (HPE) and culture. The rate of positivity of *H. pylori* in the biopsies by HPE, culture and RUT were 42.1%, 39.5% and 36.9% respectively.

RUT was easy to perform, less expensive and more rapid when compared to histopathology and culture. The rate of positivity for *H. pylori* is more by HPE than Culture and RUT and this may be due to some factors like delay in transportation of samples to the laboratory, organism not being present at the region where the biopsy was taken or exposure of biopsy sample to aerobic condition that resulted in *H. pylori* not growing in culture plate. The lower percentage of positivity of RUT in comparison with HPE and culture may be due to changes

Table 3: Distribution of *cagA* and *vacA* genotypes.

Gene (N=15)		<i>cagA</i>	
		Positive	Negative
<i>vacA</i>	s1m1	8 (53.3%)	1(6.7%)
	s1m2	6(40%)	-
	s2m2	-	-

in pH of the gastric mucosa that would have led to less sensitivity of the RUT [13].

Studies on *H. pylori* have shown that there are two important virulence factors - *vacA* and *cagA* genes. Both genes are involved in production of two distinct toxins which is able to affect cell shape and immune cells and may involve in severe gastric disease [14]. The amount of toxins produced corresponds with the several alleles of *vacA* genotypes where s1m1 produces more toxin, s1m2 produces moderate toxin and s2m2 produces few or no toxin. Heterogeneity of *vacA* alleles may be an important factor in understanding variations in clinical manifestations [15]. Rate of virulence genes harbored by strains isolated from this part of Karnataka showed 90% positivity for *cagA* genes. The polymorphic nature of *vacA* alleles were also studied and were found to be s1m1 (60%), s1m2 (40%) and no strains harboring s2m2 genotypes.

Helicobacter pylori, is known to harbor virulence genes which are implicated in gastric epithelial damage including gastric mucosal atrophy. Though it exists as a colonizing flora in some proportion of the individuals, it is essential to screen and identify those harboring the virulent pathotypes so that their progression to gastric and related carcinomas can be prevented. This study provides important information regarding the rate of identification of *H. pylori* using RUT, HPE, Culture and also their comparison and rate of virulence genes harbored by strains isolated from this part of Karnataka. Since the present work is a preliminary work with small sample size we were unable to correlate the virulence factor with disease pattern. Further analysis of strain diversity and its association with clinical disease is needed to clarify the pathogenicity of *H. pylori*. The above work is first of its kind on *Helicobacter* from this part of the country.

References

1. Kusters JG, van Vliet AH, Kuipers EJ. Pathogenesis of *Helicobacter pylori* infection. *Clin Microbiol Rev.* 2006; 19: 449-490.
2. Amieva MR, El-Omar EM. Host-bacterial interactions in *Helicobacter pylori* infection. *Gastroenterology.* 2008; 134: 306-323.
3. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet.* 1984; 1: 1311-1315.
4. [No authors listed]. An international association between *Helicobacter pylori* infection and gastric cancer. The EUROGAST Study Group. *Lancet.* 1993; 341: 1359-1362.
5. Lindkvist P, Asrat D, Nilsson I, Tsega E, Olsson GL, Wretling B, Giesecke J. Age at acquisition of *Helicobacter pylori* infection: comparison of a high and a low prevalence country. *Scand J Infect Dis.* 1996; 28: 181-184.
6. Sitas F, Yarnell J, Forman D. *Helicobacter pylori* infection rates in relation to age and social class in a population of Welsh men. *Gut.* 1992; 33:1582.
7. World Gastroenterology Organisation Global Guidelines. *Helicobacter pylori* in developing countries August 2010.
8. Misra V, Pandey R, Misra SP, Dwivedi M1. *Helicobacter pylori* and gastric cancer: Indian enigma. *World J Gastroenterol.* 2014; 20: 1503-1509.
9. Zabaleta J1. Multifactorial etiology of gastric cancer. *Methods Mol Biol.* 2012; 863: 411-435.
10. Chattopadhyay S, Datta S, Chowdhury A, Chowdhury S, Mukhopadhyay AK, Rajendran K, et al. Virulence genes in *Helicobacter pylori* strains from West Bengal residents with overt *H. pylori*-associated disease and healthy volunteers. *J Clin Microbiol.* 2002; 40: 2622- 2625.
11. Chattopadhyay S, Patra R, Ramamurthy T, Chowdhury A, Santra A, Dhali GK, B et al. Multiplex PCR assay for rapid detection and genotyping of *Helicobacter pylori* directly from biopsy specimens. *J Clin Microbiol.* 2004; 42: 2821-2824.
12. Essawi T, Hammoudeh W, Sabri I, Sweidan W, Farraj MA. Determination of *Helicobacter pylori* Virulence Genes in Gastric Biopsies by PCR. *ISRN Gastroenterol.* 2013; 2013: 606258.
13. Ricci C, Holton J, Vaira D. Diagnosis of *Helicobacter pylori*: invasive and non-invasive tests. *Best Pract Res Clin Gastroenterol.* 2007; 21: 299-313.
14. Marc Roger Couturier. The Evolving Challenges of *Helicobacter pylori* Disease, Diagnostics, and Treatment, Part 1. *Clinical Microbiology Newsletter.* 2013; 35 : 19-24.
15. Jones KR, Whitmire JM, Merrell DS. A Tale of Two Toxins: *Helicobacter Pylori CagA* and *VacA* Modulate Host Pathways that Impact Disease. *Front Microbiol.* 2010; 1: 115.