DETECTION OF *HELICOBACTER PYLORI* USING NESTED POLYMERASE CHAIN REACTION IN GASTRIC BIOPSY SAMPLES

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ABSTRACT

Helicobacter pylori remains a controversial organism with regards to humans, its epidemiology still unclear nearly two decades after discovery. The present study was undertaken to estimate the prevalence of the organism in the gastrointestinal tract in symptomatic and asymptomatic subjects to understand its precise natural history in India. A total of 154 specimens were a part of the study. These included gastric biopsies from peptic ulcer disease and Non ulcer dyspepsia subjects, as visualized on endoscopy, saliva and stool samples from apparently normal healthy adults. Nested polymerase chain reaction was performed using the primers Hp1, Hp2, Hp3 targeting 16S rRNA gene. A prevalence of 65.1%, 100%, 66.7%, and 73.3% respectively was observed by polymerase chain reaction. No association was observed between the *H.pylori* status and the disease condition of the patient.

KEY WORDS

Helicobacter pylori, Polymerase chain reaction, Peptic ulcer disease, Non ulcer dyspepsia.

INTRODUCTION

H. pylori, first isolated by Warren and Marshall in 1983, is now known to be one of the most common bacterial infections in humans (1). The prevalence of *H.pylori* differs significantly, both between and within countries, with high rates of infection being associated with low socioeconomic status and overcrowding. In developed countries, very few infections occur during childhood and a gradual increase in prevalence is observed with age, leading to infection rates of about 50% at 50-60 years. In developing countries the prevalence may be as high as 90% in adults probably because most people in these countries are infected in childhood (2). Although in terms of absolute numbers, *H.pylori* related diseases are quite high, but it appears that *H.pylori* is responsible for less morbidity from acid peptic diseases in India than its widespread prevalence would demand (3).

H.pylori can be diagnosed by a variety of methods. Urease based tests like Rapid Urease Test, though, non-specific, can be used to screen biopsy tissue for *H.pylori* infection since its sensitivity approaches 100% in multiple studies and is cost effective. Culture methods might underestimate the prevalence because of the fastidious nature of the organism. Polymerase Chain Reaction (PCR) has come up as one of the most reliable methods for detection of the organism because it enables specific detection of a very small number of nucleic acid molecules and its high sensitivity makes it the most appropriate method for diagnosis when the organism is present in low numbers, is slow growing or is difficult to identify in body tissues. Various targets have been used for DNA amplification such as Urease A gene (Ure A), Urease C gene (Ure C), 16S rRNA gene and a randomly selected sequence of chromosomal DNA, the 26 KDa species-specific antigen gene and the 0.86 KB gene of *H. pylori*, with varying results(4). Bacterial 16S r RNA was chosen as it has highly conserved regions and multiple copies. Primers used were, Hp1, Hp2 and Hp 3 in a 2-step amplification that resulted in a 109 bp amplicon which could be visualized by agarose gel electrophoresis. To the best of our knowledge, no detailed study regarding the prevalence of *H.pylori* in India, as detected by PCR, has been reported. The objectives of this study, therefore, were to know the prevalence of *H.pylori* in the

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gastrointestinal tract (GIT) of patients with ulcer and non ulcer dyspepsia and also in specimens like saliva and stool from apparently healthy individuals using nested PCR so as to try and comprehend the true nature of the host-organism relationship in the Indian population.

MATERIALS AND METHODS

This prospective study was undertaken in a tertiary care hospital in the eastern part of northern India from May 2003-Feb 2004. Patients presenting to the Gastroenterology OPD with dyspeptic symptoms such as pain in abdomen, nausea, vomiting, heartburn and belching, were enrolled in the study (Group I) after informed consent. Patients with history of intake of antibiotics at least 4 weeks prior to the collection of the specimen were excluded from the study. All patients enrolled were further categorized based on endoscopic findings. Mucosal breaks ≥ 5 mm in size with apparent depth were diagnosed as ulcer. Those found to have an ulcer (Duodenal/Gastric) were termed Peptic Ulcer Disease (PUD) cases and placed in Group Ia, n=43. Those not found to have an ulcer were termed Non Ulcer Dyspepsia (NUD) patients and placed in Group Ib, n=51. Stomach biopsies, from antrum, at least 6 in number (due to patchy distribution of the bacteria) were collected from these 94 subjects using aseptic precautions. The endoscopes were fully disinfected manually using 2% glutaraldehyde. Samples were stored in normal saline at 4°C till further processing.

All the specimens were used for the extraction of DNA. Isolation was done using standard proteinase K, phenol chloroform method (5). Each patient's group of 5 specimens underwent DNA extraction in a single batch accompanied by a negative control tube to check contamination from laboratory or cross contamination among specimens. Saliva, 8-10ml each and stool, 1-2g each, collected from Healthy volunteers served as controls (Group II), n=30. In view of high content of PCR inhibitors in stool samples the extraction was made more

rigorous as per Van Zwet et al (6) with slight modifications. Isolated DNA was stored at -20°C till further use.

A nested PCR reaction test for 16S rRNA gene of *H. pylori* as described by Ho et al (5) and Mapstone et al (7) was performed using the following primers-Hp1: CTG GAG AGA CTA AGC CCT CC position 834-853, Hp2: ATT ACT GAC GCT GAT TGT GC position 744-763, Hp3: AGG ATG AAG GTT TAA GGA TT position 407-426. The first PCR, using Hp1 Hp3 amplified a 446 bp fragment of the gene encoding *H. pylori* 16S rRNA. It consisted of 30 cycles at an initial denaturation of 95°C for 3 min; 95°C for 30 sec (denaturation); 55°C for 30 sec (annealing) and 72°C for 30 sec (extension) followed by 72°C for 7 min (final extension). The reaction mixture, 25µl, contained 2.5µl 10 X buffer, 1.1µl 25mM MgCl₂, 10pmol of each primer, Hp1 Hp3, 1µl dNTP mix and 1U Taq DNA polymerase (all from GENE I). Approximately 100ng of the isolated DNA was used as template. De-ionized water was added to make up the reaction volume.

The profile of the second round was similar to the first except that the annealing temperature was raised to 62°C, 25 pmoles each of Hp1 and Hp2 were used and 0.3µl of the first round product served as template. Positive and negative controls were included for each batch of amplification. The DNA from *H. pylori* strain J99 provided by Francis Megraud, Centre National de Reference des Campylobacters et Helicobacters. Laboratoire de Bacteriologie, Hopital Pellegrin, 33076 Bordeaux Cedex, France, served as positive control. De-ionized water was used as negative control. Presence of PCR inhibitors in the sample was ruled out by using spiked samples. The final PCR product of 109 bp, was visualized under ultra violet light after 2% agarose gel electrophoresis with ethidium bromide staining.

RESULTS

A male preponderance was seen in the patients (79.1%, PUD and 62.7%, NUD). Majority of the patients were in the 31-60 years age group. (69.8%, PUD and 70.6%, NUD). The most common presenting feature was pain abdomen, 97.7% in PUD and 96.1% in NUD patients. Duodenal ulcers were more common (62.7%) than gastric ulcers (37.2%). Moreover single ulcer was found more frequently. The positivity, by PCR, in antral biopsies in the NUD (100%) patients was significantly higher ($p < 0.01$) than the PUD patients (65.1%). In healthy volunteers, who served as controls, a positivity of 66.7% and 73.3% was seen in saliva and stool samples respectively (Table 1).

Table 1 : PCR positivity for *H. pylori* in study and control groups

Group			Specimen	Total number(n)	Positive for <i>H. pylori</i> by PCR	Percentage
I	Ia	PUD	Stomach biopsy	43	28	65.1%
	Ib	NUD	Stomach biopsy	51	51	100%
II	Healthy Volunteers		Saliva	30	20	66.7%
			Stool	30	22	73.3%

DISCUSSION

Long held view that human stomach is sterile owing to its acidity is now being challenged. Recent evidence suggests that microorganisms have probably co-evolved with the humans (8). On one hand reports from across the globe suggest close association of *H.pylori* with gastric diseases. On the other, studies suggest fewer than 20% infected patients develop any clinical consequences (9).

In our study, the nested PCR, targeting 16SrRNA, yielded a positivity of 65.1% in the stomach biopsies of PUD patients as against 100% in NUD patients. This observation questions the disease producing ability of *H. pylori*. The organism was also detected in saliva (66.7%) and stool (73.3%), in healthy volunteers. *H.pylori* in saliva of healthy volunteers points towards oral cavity as an important reservoir. Studies both support and contradict this. It has been reported that dental plaque may harbor more than 350 different species and *H.pylori* like organisms in the micro flora (10) and that *H.pylori* may belong to the normal oral micro flora. Detection of the organism throughout GIT at such a high frequency strongly suggests that it may be a commensal and not a pathogen as very firmly believed and acted upon by using aggressive antibiotic therapy in dyspeptic subjects.

Loffeld et al (11) suggested, as early as 1990, that disruption of the balance between the host defense and the virulence of the microorganism is probably responsible for gastric diseases. Monstein et al (12) too, have reported that bacteria other than helicobacter do colonize the stomach and suggested that, in the absence of gastric inflammation, helicobacter could be a part of normal flora of stomach.

Further, occurrence of acid peptic disease in only 20% of infected individuals and non-association of the bacterium with gastric adeno carcinoma (13), especially in Indian subjects, supports its role as a commensal. Strain variation and age of exposure, leading to varying spectrum of illness cannot be denied. It has been said that *H.pylori* may have different consequences in different populations because of variation in strain, host, or environmental cofactors (14).

To conclude, the presence of this bacterium in the stomach and other parts of GIT suggests that *H.pylori* is a part of normal biota, and that some strains could be more virulent than others and host factors may be responsible for disease causation. Correlation of strain variability in relation to body site, disease and geographic area may result in therapeutic decisions that will improve the outcome of patients infected with *H.pylori*.

REFERENCES

1. Goodwin CS, Mendall MM, Northfield TC. *Helicobacter pylori* infection. Lancet 1997; 349:265-9.
2. Megraud F, Brassens-Rabbe MP, Francois D, Aouicha B, Duong QH. Seroepidemiology of *Campylobacter pylori* infection in various populations. J Clin Microbiol 1989; 27:1870-3.
3. Abraham P, Bhatia S. Position paper on *H.pylori* in India. Ind J Gastroenterol 1997; 16 (1) S29-S33.
4. Kabir S. The role of PCR in the diagnosis of *H.pylori* infections. Rev Med Microbiol 1999; 10(4): 197-212.
5. Ho SA, Jane A, Lewis FA, Secker AD, Cross D, Mapstone NP et al. Direct Polymerase Chain Reaction test for detection of *H.pylori* in humans and animals. J Clin Microbiol 1991; 29(1): 2543-9.
6. Van Zwet AA, Thijs JC, Kooistra-Smid AMD, Schrim J, Snider JAM. Use of PCR with feces for detection of *Helicobacter pylori* infections in patients. J Clin Microbiol 1994; 32: 1346-8.
7. Mapstone NP, Lynch DAF, Lewis FA, Axon ATR, Tompkins DS, Dixon MF, Quirke P. Identification of *Helicobacter pylori* DNA in the mouth and stomach of patients with gastritis using PCR. J Clin Pathol 1993; 46: 540-3.
8. Blaser MJ. Hypothesis: The changing relationship of *H.pylori* and humans. Implications for health and disease. Infect Dis 1999; 179: 1523- 30.
9. Blaser MJ. Not all *Helicobacter* strains are created equal: should all be eliminated? Lancet 1997; 349: 1020- 22.
10. Song Q, Lange T, Spahr A, Adler G, Bode G. Characteristic distribution pattern of *Helicobacter pylori* in dental plaque and saliva detected with nested PCR. J Med Microbiol 2000; 49: 349-53.
11. Loffeld RJLF, Stobbersingh E, Flendrig JA, Arends JW. Presence of *H.pylori* in patients with Non-Ulcer Dyspepsia revealing normal antral histological characteristics. Digestion 1990; 47:29-34.
12. Monstein HJ, Tiveljung A, Kraft CH, Borch K, Jonasson J. Profiling of bacterial flora in gastric biopsies from patients with *H.pylori* associated gastritis and histologically normal control individuals by temperature gradient gel electrophoresis and 16S rDNA sequence analysis. J Med Microbiol 2000; 49: 817- 22.
13. Nath G, Khanna AK, Jain AK, Gulati VK. *H.pylori* does not cause gastric carcinoma in India. The Nat Med J India 2000; 13(6): 328-9.
14. Blaser MJ. Heterogeneity of *H.pylori*. Eur J Gastroenterol Hepatol 1997; 9: 83-7.