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MOLECULAR DETECTION OF *HELICOBACTER PYLORI* IN PUD GASTRIC BIOPSY SAMPLES WITH REFERENCE TO VIRULENT GENES

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ABSTRACT

Helicobacter pylori is a known agent associated with Peptic ulcer disease(PUD), however the isolation and identification of *H.pylori* using conventional methodologies are challenging, hence a study was conducted to isolate *Helicobacter pylori* from biopsy samples of patients with PUD (Peptic ulcer disease) on selective agar media after the preliminary (Campylobacter like organism) CLO testing and genotyping using genus specific 16SrRNA and characterization of virulent factors viz. cagA, vacA and flagella A and nap A which are the predominant among the isolated clinical strains of *Helicobacter pylori.*The rate of positive *H. pylori* in the collected biopsies after the CLO test was 83% after amplification with genus specific 16SrRNA of *Helicobacter pylori*. The rates of the virulence genes cagA, vacA were 79.4% (62/78) and 25.6% (20/78), respectively. Seven patients were found colonized with both genotypes (cagA + vacA) having a rate of (3%). cagA status in association with vacA genotype has been implicated as a high risk factor for *H. pylori* related gastric pathologies. flagellaA, and nap A were found to be prevalent in all the positive samples of *H.pylori*. Fresh biopsy samples which were subjected to culture media containing antibiotics could obtain only few *H.pylori* isolates. Therefore a genotypic identification of 16sRNA was found to be highly sensitive for the detection of *H.pylori* than a bed side CLO test which could be positive for other Co-harboring bacterial species.

KEYWORDS: Clinical specimen, Peptic Ulcer Disease (PUD), Virulent genes, Cytotoxin associated gene, Vaculation associated cytotoxin ,

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INTRODUCTION

Helicobacter pylori has coexisted within humans for more than thousands years, it was found that 80% of population infected with *H.pylori* are asymptomatic as it adapts itself to the natural ecology of human stomach. [1] According to the World Health Organization (WHO) the bacterium was recognized as a type I carcinogen, and now it is considered the most common etiologic agent of infection-related cancers. *H. pylori* infection is a major risk factor for peptic ulcer disease (PUD) and is responsible for the majority of ulcers of the stomach and upper small intestine and is considered as the primary infection when untreated leads to the formation of severe complications towards the formation of adenocarcinoma.[2]The adhesions which are virulent bacterial surface proteins play a key role for the adherence of bacterium onto the mucosal epithelial cells. The pathogenesis, inflammation and the colonization of the bacterium are associated with major virulent factors such as Cytotoxin associated gene (cag A) and Vacuolating cytotoxin gene (vac A). *H. pylori* strains isolated from gastric epithelium can be categorized in two groups, viz type I which has cagPAI (cag pathogenicity island) which are mostly coupled to the ability to cause ulceration and a positive correlation to form adenocarcinoma[3] in comparison to the less virulent type II strains which does not have cag PAI region.[4]The primary virulence factors associated with *H. pylori* is vacA gene. It is an oligomeric toxin composed of 87 kDa active subunits. An antiserum produced against these purified proteins neutralizes the cytotoxic activity that activates vacA and induces an inactivation of energy metabolism followed by mitochondrial damage, leading to impairment of the cell cycle in gastric epithelial cells.[5] The presence of flagella is an essential factor of colonization in *H. pylori*. flagellin proteins usually contribute to persistent motility and infection of the gastric mucosa.[6]*H. pylori* play a key role in the activation of neutrophils to increase neutrophil adherence to endothelial cells through the expression of an activating protein (Hp-nap.[7] Hp-nap induces adhesion of neutrophils to endothelial cells and production of reactive oxygen radicals, detected by nitro-benzo-tetrazolium reduction.[8]The aim of the present study is focused on the isolation of *Helicobacter pylori* from biopsy samples of patients with PUD (Peptic ulcer disease) on selective agar media after the preliminary (Campylobacter like organism) CLO testing and genotyping using genus specific 16sRNA to detect colonization of *H.pylori* among other Co harbored bacterium by direct PCR using gastric biopsy samples and characterization of virulent genes which are the predominant among the isolated clinical strains of *Helicobacter pylori*.

MATERIALS AND METHODS

Collection of clinical specimen

A total of 124 non-repetitive gastric biopsy samples from March 2013 were collected from two different tertiary care hospitals subjected to Campylobacter-like organism (CLO) test which is a rapid diagnostic test commonly followed by the Gastroenterologist for *Helicobacter pylori* identification. The principle of the test is the ability of *H.pylori* to secrete the Urease enzyme, which catalyzes the conversion of urea to ammonia and carbon dioxide. The samples which were positive for CLO test was directly used for DNA isolation and another fresh sample from the same patients were collected in Wang's semisolid transport media (Hi-media) for isolation of *Helicobacter pylori*.

Isolation of Helicobacter pylori from fresh biopsy samples

As per standard procedure the biopsy specimens were directly inoculated in Brain heart infusion (BHI) agar plates supplemented with 7% sheep blood, vitamin 10ml/L, new born calf serum 100 ml/L, antibiotics such as vancomycin hydrochloride (10 mg/ml), trimethoprim lactate (5 mg/L), amphotericin-B (5 mg/ml) and incubated in anaerobic atmosphere using anaerobic gas pack (Hi-media) at 37°C for 5-7 days. The isolates later were identified as *H. pylori* by Gram staining, colony morphology, positive Urease, Oxidase and Catalase reactions.

DNA Extraction from biopsy

Reference strain of *Helicobacter pylori* 26695 provided by "National Institute of Cholera and Enteric Diseases (NICED)" was used as control for molecular studies. DNA was extracted from biopsies by Mechanical shearing and Thermal shock method, by boiling in 100 µl of sterile double distilled water for 10 min and cooling it on ice for 5 min. It was then centrifuged at 10,000 rpm for 10 min. The supernatant containing DNA was used of PCR study.[9]

PCR amplification of Helicobacter pylori 16S rRNA

Genomic DNA extracted from biopsies and also from the isolates were subjected to PCR using *H. pylori* 16S rRNA specific primers ["Hp16s"] (Sigma, India) The PCR conditions used were as follows: Initial denaturation at 95°C for 7mins, 30 cycles of 95°C for 50s, 56°C for 1min and 72° C for 1 min and an extension time of 72°C for 5 min. The amplified genes were detected by electrophoresis in a 1% agarose gel with ethidium bromide and bands visualized using UV transilluminator (Table 1, Figure 1a).

Genotyping of H. pylori virulent genes

The primers for virulent genes *cagA, vacA* , *nap* A and *fla* A (Sigma , India) were used for this study (Table 1). PCR was performed using 2 µl of DNA solution to a final volume of 25 µl containing, 10X buffer 1.5 mM MgCl2, 250 µM dNTPs, 10 pico moles each of forward and reverse primers and 1 U of Taq DNA polymerase in a

conventional Thermocycler. Amplified products were separated by agarose gel (1%) with ethidium bromide and bands visualized using UV transilluminator (Table 1, Figure1 b, c, d, e)

RESULTS

Among 124 gastric biopsy samples which were collected by endoscopy from the PUD (peptic ulcer disease) patients suffering from mild to chronic gastritis 89 samples were found positive after the preliminary CLO test. *H. pylori* isolates were transparent colonies, Gram negative-rod shaped Urease, Catalase and Oxidase positive and was obtained only from 4 fresh biopsy samples which could be due to Co-harboring contaminants despite inclusion of multiple antibiotics in the culture medium or bacterial cells failing to survive further subculture. The time taken for transport of samples is also an important criteria on the isolation of *Helicobacter pylori*. The bed side rapid Urease test(CLO test) followed by the gastroenterologist is found to be non specific identification to *H.pylori* as there are other species which were found to be on the normal ecological niche that can detect Urease false positive for the same test. The samples processed had other co-harboring bacteria such as *Pseudomonas*, *Campylobacter*, *Bacteroides*, *Eubacterium*, and *Staphylococcus*-like bacteria which grow quite faster and suppress the growth of *H.pylori*.

Genotyping of 16rRna Helicobacter pylori

89 biopsy samples which were positive by CLO test were subjected to the *Helicobacter pylori* 16SrRna PCR with genus specific **"**Hp16Sr RNA" primers, Only 78 was found positive by specific primer and the amplicon size was 109 bp. (Figure 1a)

vacA and cagA genotypes of Helicobacter pylori colonized in gastric mucosa

Screening of virulent genes such as vacA, cagA was done associated with the pathogenesis and prevalence among the clinical samples. The rates of the virulence genes cagA, vacA were 79.4% (62/78) and 25.6% (20/78), having amplicon size of 254bp and 400bp respectively. Out of which, seven patients colonized with both genotypes were 3% (cag A + Vac A). cagA status in association with vacA genotype has been implicated as a high risk factor for *Helicobacter pylori* related gastric pathologies. The rate of virulence genes cagA, vacA distribution among the PUD patients is summarized in (Table2; Figure 1b,c)

flagella region and neutrophill associated protein region

Flagella region has both fla A and fla B subunits and fla A was most predominant in all the strains of *Helicobacter pylori* and it is one of the virulent factor where as flagellum-dependent motility and chemotaxis are crucial factors in the process of colonization in the host organism and establishment of a successful infection. We have screened fla A and it was found in most of the clinical samples showing 99% prevalence and yielding the product size of 500bp.*Helicobacter pylori* is accompanied by a large infiltration of the mucosa by neutrophils, which are believed to contribute substantially to *H. pylori*–induced gastritis. In our study all the clinical samples had nap A gene yielding the product at 370bp, which shows that this gene plays a major role on the inflammatory cell response. Thus activates human neutrophils followed by an increased expression of neutrophil and increased adhesiveness to endothelial cells. (Figure1 d,e)

Table 1

Different specific primers used for the identification of Helicobacter pylori genes

H.Pylori gene	Nucleotide sequence	Annealing temperature(°C)	Reference
Hp(16srRNA) (Specific for *Helicobacter pylori*)	**Hp1Forward**5'CTGGAGAGACTAAGCCCTCC3' **Hp2Reverse**5'ATTACTGACGCTGATTGTGC-3'	56	Ho et al., 1991
	VIRULENT GENES		
cag A	**Forward**5'GATAACAGGCAAGCTTTTGAGG 3' **Reverse** 5'CCATGAACTTTTGATCCGTTCGG 3'	58	NCBI database
vac A	**Forward** 5' ACAAACACACCGCAAAATCA 3' **Reverse** 5' CCTCTGCCTGCTTGAA 3'	55	NCBI database
nap A	**Forward** 5' TGCAAGCGGATGCGATCGTGTT3' **Revers**5'GCAACTTGGCCAATTGATCGTCCGC3'	60	Evans et al.,1995
fla A	**Forward** 5' TTCTATCGGCTCTACCAC3' **Reverse** 5' CTGACCGCCATTGACCAT3'	55	Day et al., 2000

Table2
Genotypic percentage of distribution of vac A and cag A gene among different PUD patients

Patient group	No. (%) positive for:	
	cagA	vacA
G (*n* = 37)	32 (86)	3 (8)
GU (*n* = 15)	9 (60)	6 (40)
DU (*n* = 26)	21(81)	11 (42)
Total (*n* = 78)	62 (79.4)	20 (25.6)

Abbreviations: G, gastritis; GU, gastric ulcer; DU, duodenal ulcer; n, number examined.

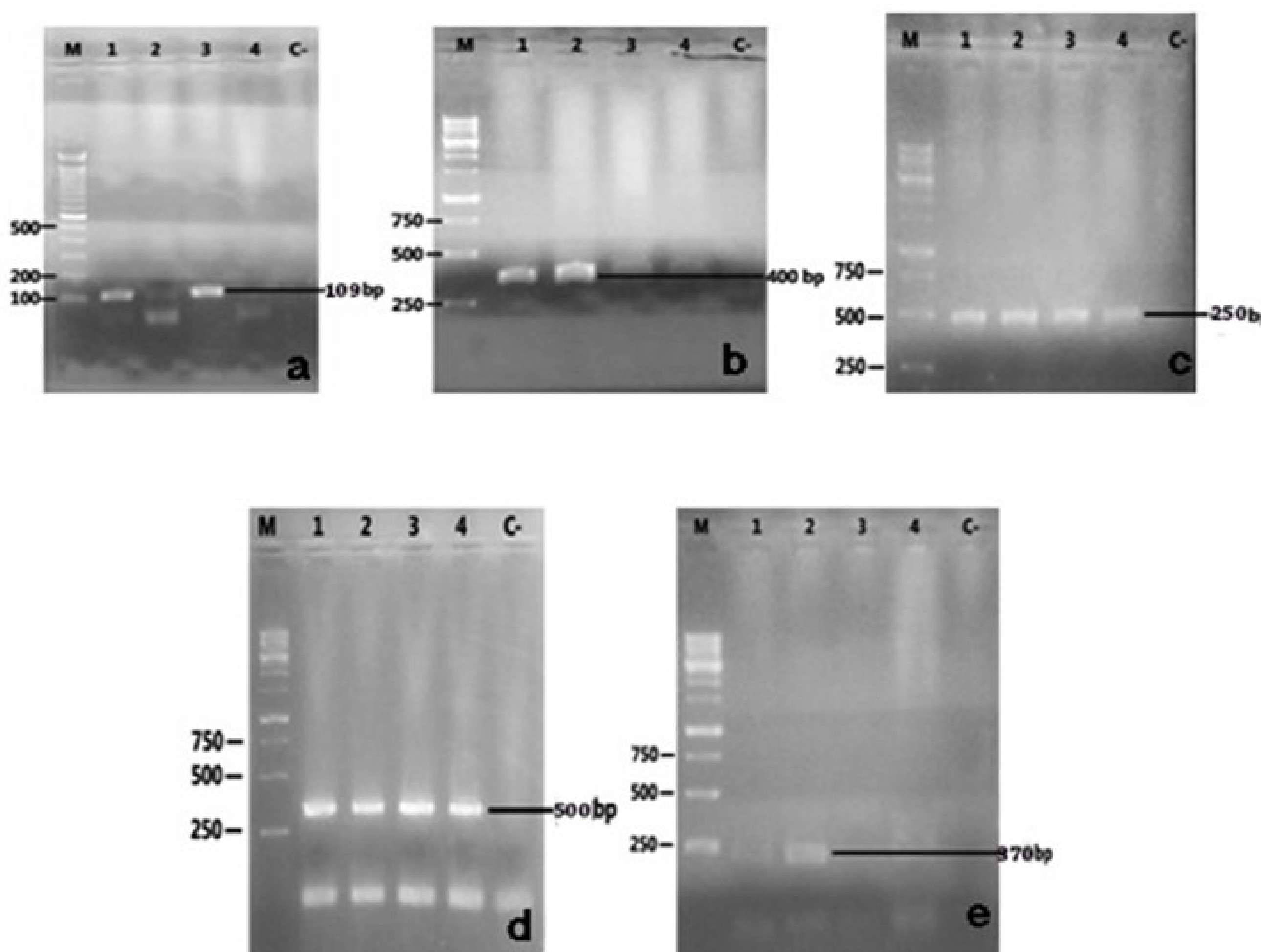

Figure1
PCR amplification of Helicobacter pylori genes (a) 16s rRNA gene (b) cagA gene (c) vacA gene (d) fla A gene (e) nap A from gastric mucosal biopsies collected from PUD (peptic ulcer disease) patients. The PCR products were electrophorsed on 1.5% agarose gel and visualized by ethidium bromide staining.

DISCUSSION

The success in culture and isolation of this organism is influenced by various factors analogous to the biopsy site preparation, collection and transport of the specimen and the culture media used for isolation. Moreover, isolation of *Helicobacter pylori* from mucosal biopsy samples is a pre-requisite for further studies of the organism including susceptibility testing, analysis of virulence factors and molecular epidemiological studies. High incidence of false negative CLO test result in our study suggests that CLO alone might not be a reliable test.[10-13]The primers analyzed in this study to detect 16SrRNA genes have a highly species specific probing for the detection of *H. pylori* with high specificity and sensitivity as indicated by their inventors [14-16], and due to cultural and immunological methods difficulties, the direct PCR of biopsy samples will give this method a great importance in detection and subsequent eradication follow up of *H.pylori*.[17-19] *H. pylori* infected dyspepsia patients from Kozhikode area of Kerala state reported high risk of (vacAs1+cagA) genotype combination which was found in (14%) [9] where as our study showed lesser rate (3%) of correlation of vac A with cag A gene, among the PUD (peptic ulcer disease) patients which ascertains that the risk factor of gastric adenocarcinoma could be considerably less. The prevalence of cagA positive *H.pylori* varies from one geographic region to another. The cag A results in our study of 79.4. % is higher to those obtained in Tunisia of 61.6% and Pakistan (56%),[20-22] similar to reports obtained in Iran (76%) and Iraq (71%).[23] In Calcutta, India, PCR tests indicated that 80–90% carried the cagA pathogenicity island and the potentially toxigenic vacA s1 alleles of the vacuolating cytotoxin gene vac A independent of disease status [24], which was noted to be higher in rate than our study. No significant variation was identified in the *napA* sequence between *H. pylori* isolates that activate neutrophils and those that do not. The *napA* sequence was highly conserved and present in all isolates[25] A study which was conducted at Baghdad University Iraq showed 99% similarity of identification of flagallin A gene with 16sRNA*.*[26] was analogous to the present study.

CONCLUSION

The culturing of biopsy samples after the rapid in-house CLO testing cannot be 100% relayed on due to false positivity of other harboring bacterium Therefore genotyping with genus specific 16SrRNA rapidly allow treatment to be designed for the individuals, and will be more effective and result in fewer side effects, reduce overall costs, and lead to a decrease treatment failures and development of fewer antibiotic-resistant. Our study reported the prevalence of the virulent genotypes of *H. pylori* isolates from in and around Coimbatore region of Tamilnadu. However, the multicentre and large scale surveillance studies are required to help us better understand epidemiological significance of this disease and the association between *H. pylori* genotypes and clinical outcome in different regions and populations. This study also encourages us to look for a possible cag A based vaccine candidate to treat gastric cancer.

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