International Journal of Medical Research Professionals
P-ISSN: 2454-6356; E-ISSN: 2454-6364
DOI: 10.21276/ijmrp

IJMRP

Original Research Article

Genetic Diversity among Clinical Isolates of *Helicobacter Pylori* Detected From India

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ABSTRACT

Background: *Helicobacter pylori* is a pathogenic gram negative bacteria found in human stomach. It is present in almost 50% of human population worldwide and its infection is linked to development of various diseases ranging from gastrointestinal disorders to gastric cancer. The objective of the present study was to assess the epidemiology and genomic variability of *H. pylori* strains prevalent in patients of Jammu (J&K).

Methods: Mix of qualitative case study methods (key informant interviews, document review and structured questionnaires) was used to study epidemiology. Clinical biopsy specimens were collected from the patients undergoing gastrointestinal endoscopy. The DNA diversity was determined by RAPD and multiplex PCR.

Results: 40 patients were found *H. pylori* positive out of which 64% were males and 36% were females. Subjects who were in the age group of 36-45 years, smokers, alcohol consumers, non-vegetarians were found to be associated with this infection. Abdominal pain was the most frequent symptom. Primer 1283 was more sensitive compared to 1254 and 1281 in differentiating infections in our patients. Most of the patients were cagA positive.

Conclusions: The preliminary results obtained in this study indicate that RAPD markers may show requisite polymorphism in order to assess the Genetic variability among various *H. pylori* strains. Detailed characterization of virulence genes and their allelic types may prove highly beneficial for eradication therapies of *H. pylori*.

Keywords: *Helicobacter pylori*, RAPD PCR, Jammu, India, *cagA*.

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Article History:

Received: 20-12-2016, **Revised:** 06-01-2017, **Accepted:** 09-01-2017

Access this article online	
Website: www.ijmrp.com	Quick Response code
DOI: 10.21276/ijmrp.2017.3.1.017	

INTRODUCTION

Helicobacter pylori is an important gastric pathogen chronically infecting billions of people worldwide.[1,2] Rates of infection appear to be higher in the developing rather than the developed countries.[3,4] The World Health Organization (WHO) classified *H. pylori* as a group I carcinogenic factor in 1994.[5] It owes its name to its shape- *helico* for spiral and *pylori* from gateway between stomach and intestine where this bacterium is usually found.[3] The clinical manifestations of *H. pylori* infection ranges from chronic gastritis to gastric malignancies, iron deficiency anaemia, vitamin B12 deficiency etc.[5,6] Prevalence of *H. pylori* varies with age, race, geographical location, socio-economic status, lifestyle habits etc.[7] The infection may be symptomatic or remain non-symptomatic for years.[8] The ability of *H. pylori* to colonize and cause disease depends on many factors like strain-to-strain genetic variability in bacterial virulence factors, continuous interactions with the host, gastric environment, host's clinical profile etc.

Intensified research has been conducted in the recent years on the pathogenicity factors associated with *H. pylori* infection. Antigen *Cag* A is a virulence factor of *H. pylori* which is encoded by the gene *cag* A. Its presence is associated with increased secretion of interleukin 8 (IL-8), tyrosine phosphorylation of host cells, activation of signal cascade in ERK/MAP pathway.[9] It has been reported that patients infected with *cag* A$^+$ strains have higher risk of developing gastric cancer.[10] The vacuolating cytotoxin (*Vac* A) encoded by the gene *vac* A, induces the formation of vacuoles in epithelial cells.[10] Association between virulence factor encoded by *iceA* gene and *H. pylori* infection has been firmly established. Contact between *H.pylori* and host epithelial cells activates the *iceA* gene transcription and may lead to peptic ulceration.[11] Therefore, an intricate interplay between pathogen, environment and host factors results in the progression of disease.[12] Continuous mutations and DNA

rearrangements have resulted in novel genotypes of *H. pylori*.[13] Aim of the present study was to assess the epidemiology and genomic variability of *H. pylori* strains prevalent in patients of Jammu (J&K) by molecular profiling. This is the first molecular study on this pathogen from this region.

MATERIALS AND METHODS

Subject population

Prior to commencement, the study was approved by the institutional ethical committee. The subjects were chosen from the patients attending/admitted at the Government Medical College & Hospital and a private clinic at Jammu from Oct' 2013 to Apr' 2014. After explaining the nature and purpose of study, written consent was obtained from the patients or their attendants. Cases were the patients who presented with the disease symptoms and hence subjected to gastrointestinal endoscopy. Subjects within the age group of 20-80 years; not having any previous history of cancer and residing in Jammu province for at least twenty years were considered. Individuals undergoing a) prior Helicobacter eradication therapy, b) consuming acid suppressive drugs or antibiotics in the preceding 6 months and c) having a history of operations of the upper gastrointestinal tract were excluded from the study. Mix of qualitative case study methods (key informant interviews, document review and structured questionnaires) was used. Data analysis was carried out using the statistical package for social sciences, for Windows version 16.0 (SPSS 16).

Clinical samples and culture

Two biopsies from the gastric mucosa and antrum of the stomach of each patient were obtained. All these patients underwent non-sedated upper gastrointestinal endoscopy under topical lignocaine anesthesia. One of the biopsies *i.e.* from antrum was immediately fixed in formalin solution for Giemsa staining and the other was collected in a sterile microfuge tube containing TES (TrisCl, EDTA, SDS) buffer and Proteinase K for DNA isolation. For culturing of *H. pylori*, the biopsy specimens were homogenized and cultured on Brucella Agar supplemented with sheep blood and antibiotics (vancomycin 2.0 mg, polymyxin 0.05 mg, trimethoprim 1.0 mg, and amphotericin B 2.5 mg/L). Incubation was performed in microaerophilic conditions at 37ºC for 5–7 days. Suspected isolates were identified by colony morphology, gram staining and further subjected to biochemical analysis (urease, catalase, and oxidase assays). The isolates were subcultured and stored at −70ºC for further studies.[14-16]

DNA extraction and PCR analysis

Genomic DNA was extracted using the boiling extraction method. Briefly, 300 µL of thawed isolated *H. pylori* samples were incubated at 95°C for 10 minutes. These samples were then centrifuged at 14,000 g for 10 minutes and the supernatants were stored at -20°C till further use. To check the quality of the DNA the quantification was carried out by measuring the optical density (OD) at 260nm. Isolated stock DNA was diluted with distilled water and the OD was checked at 260nm in a spectrophotometer. *Helicobacter* genus-specific 16S rDNA primers (446 bp; forward primer 5'-CTGGAGAGACTAAGCCCTCC-3', reverse primer, 5'-AGGATCAAGGTTTAAGGATT-3') were used for *H. pylori* identification. PCR program and reactions were performed as described previously by Ren *et al.*, 2012.[17]

RAPD fingerprinting

The RAPD-PCR was performed to investigate genetic relatedness and variations in *H. pylori* strains isolated from the patients. The primers used are listed in Table 1. PCR reaction mix consisted of the constituents shown in Table 2. Thermal cycling was set at 4 cycles of 5 mins at 94°C, 5 mins at 36ºC, 5 mins at 72ºC followed by 30 cycles of 1 min at 94ºC, 1 min at 36ºC, and 2min at 72ºC. The products were electrophoresed on a 1.8% agarose TAE gel and visualized on a UV-transilluminator and photographed using a gel documentation system. 1 kb ladder (Fermentas) was used as a molecular weight marker. Similarity of all RAPD banding profiles was analyzed computationally. Polymorphisms of ≤2 and ＞2 RAPD bands were considered as definitive criteria for detection of related and different strains, respectively.[1]

Table 1: List of primers used for the RAPD and multiplex PCR

Primer code	SEQUENCE
1281	5' AAC GCG CAA C 3'
1283	5' GCG ATC CCC A 3'
1254	5' CCG CAG CC AA 3'
cagA	F-GG AA CCC TAG TCG GTA ATG
	R-ATC TTT GAG CTT GTC TAT CG
iceA	F-TATTTCTGGAACTTGCGCAACCTGAT
	R-GGCCTACAACCGCATGGATAT
vacA	F:CAATCTGTCCAATCAAGCGAG
	R:GCGTCAAAATAATTCCAAGG

Table 2: Components of the PCR reaction assay

Constituents (stock)	Quantity in each tube (working)
Taq buffer (10X)	2.5µl (1X)
dNTP's (2.5 mM each)	2.5µl (0.2mM each)
$MgCl_2$ (25mM)	3.0 µl (1.5mM)
DNA (20ng)	1.0 µl (20ng)
Taq polymerase	1.0 unit
APS specific primers	5.0 pm
Deionised water	Upto 25µl

Multiplex genotyping

The multiplex-PCR genotyping were performed for vacA, iceA and cagA genes. PCR conditions were set as described previously by Farzi *et al.,* 2015.[1] Positive-control DNA samples were used in the multiplex-PCR genotyping assays.

RESULTS & DISCUSSION

The study enrolled 96 symptomatic patients of gastrointestinal disease, out of which 40 patients were found *H. pylori* positive giving a prevalence of 41.6% (Fig. 1). Among *H. pylori* positive patients, 64% were males and 36% were females (Fig. 2a). Age wise distribution showed maximum prevalence of *H. pylori* infection in the age group of 36-45 years and comparatively lower in the age groups of 26-35 and 66-78 years (Fig. 3). Abdominal pain was the most frequent symptom in 21 (52.5%) patients followed by nausea and retrosternal burning (Table 3). Endoscopic examination of *H. pylori* positive patients revealed high incidence of antral gastritis (52.5%) in patients (Fig. 4).

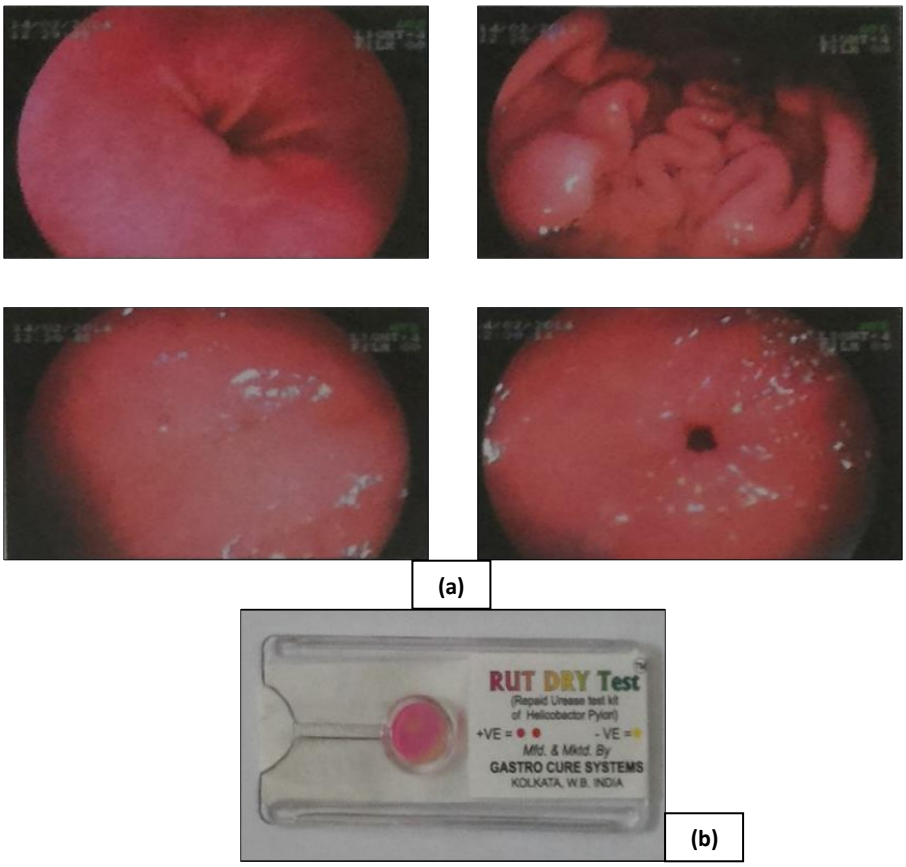
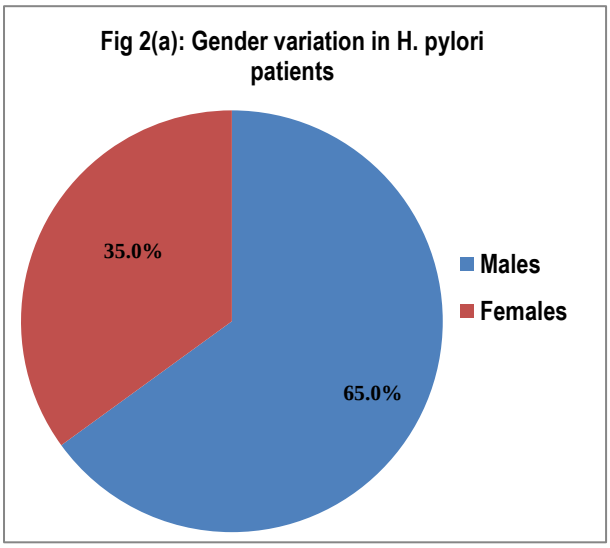


Fig 1: (a) Endoscopic images of the patients showing bile refulx (b) Slide showing positive rapid urease test.



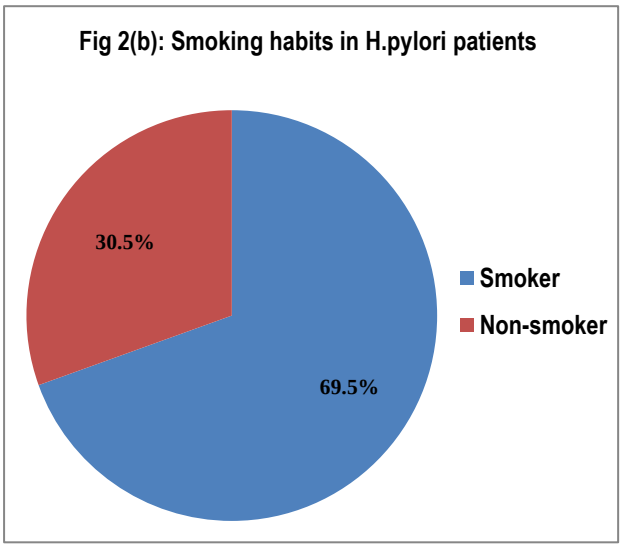


Table 3: Symptom profile of *H. pylori* positive patients

Symptom	No. of patients	Percentage
Abdominal pain	21	52.5
Nausea	9	22.5
Retrosternal burning	9	22.5
Belching	6	15
Vomiting	5	12.5
Loss of appetite	4	10

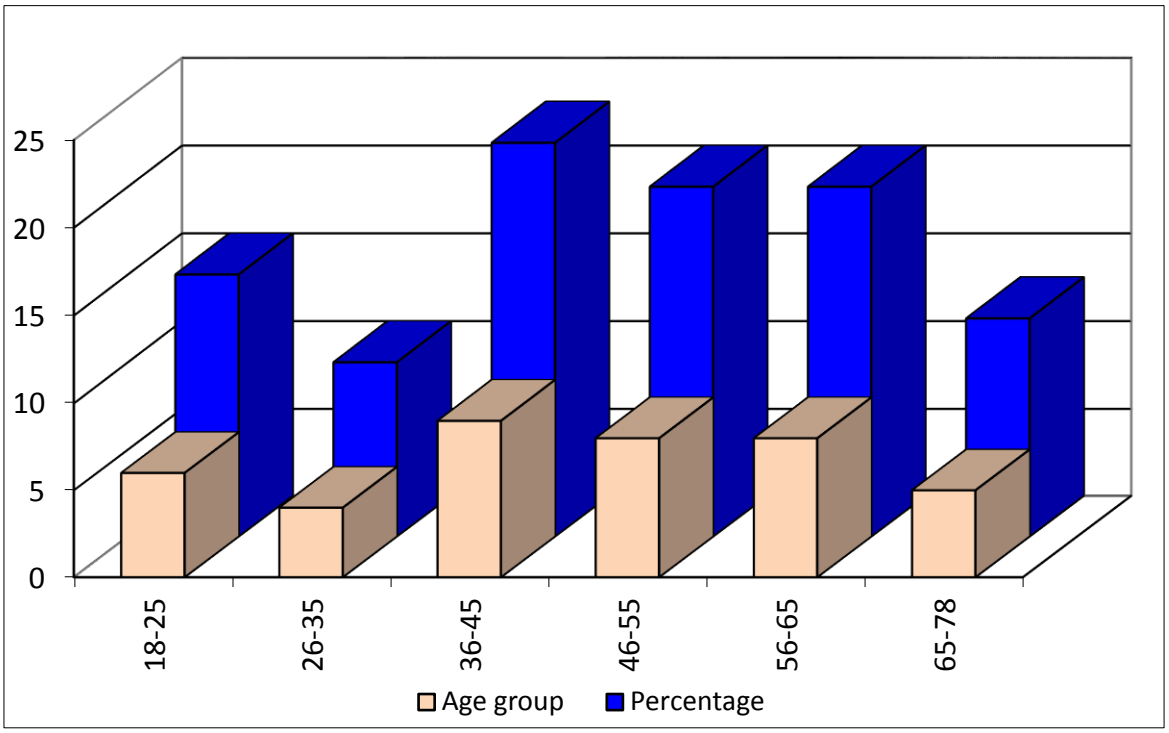


Fig 3: Age wise distribution of *H. pylori* patients

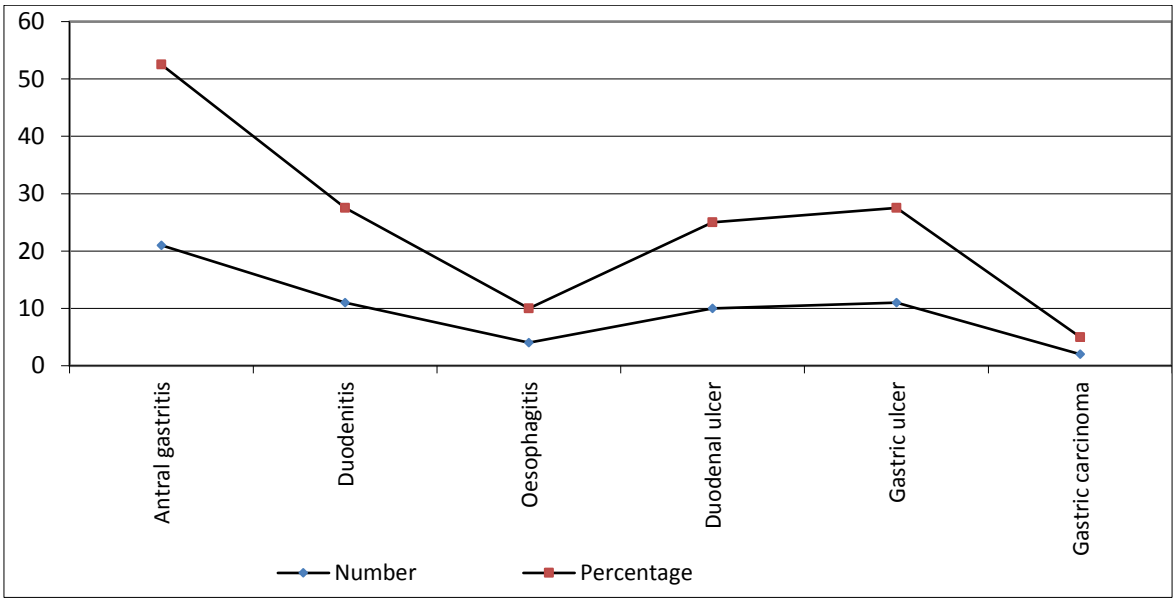
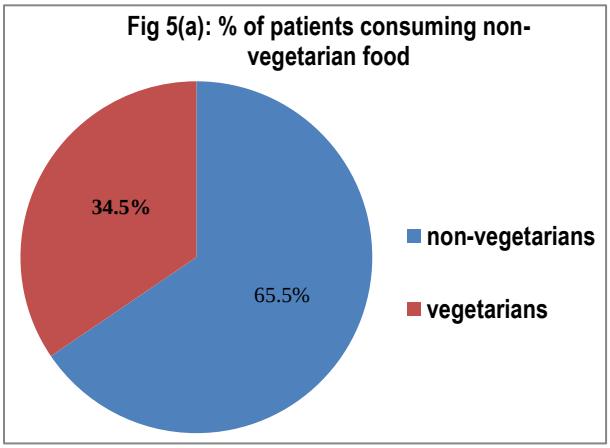
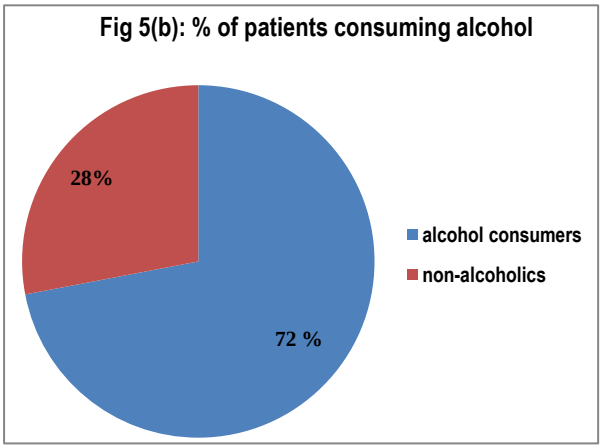


Fig 4: Endoscopic and histopathological features of *H. pylori* positive patients





There is a substantial age related prevalence of *H. pylori* in healthy asymptomatic subjects.[18] The results are in coherence with the studies reporting that healthy persons younger than 30 years have prevalence rate of about 10% whereas those over 60 years have rates approaching 60%. Smoking is another possible confiding factor which has been linked to duodenal ulcer and gastric ulcer.[19] In our study, a high rate of infection was found in patients who had smoking habits as compared to non-smokers. Percentage of smokers was 69.5% and that of non-smokers was 30.5% (Fig. 2b). It may affect the early stages of gastric carcinogenesis by inducing chronic gastritis in which there is formation of free radicals by inflammatory cells.[17] Other risk factors of *H. pylori* infection are non-vegetarian foods particularly spiced fish; which is very common in Indian diet.[20,21] In the present study, a high risk of infection was found in patient who consume non vegetarian food (65.6%) than individuals taking vegetarian diets (34.5%) (Fig 5a). In nutshell, main components which emerged as important risk factors of *H. pylori* infections in population of Jammu region were smoking, alcohol consumption (Fig. 5b), gender, age etc. although, *H. pylori* infection critically depends on specific properties of the organism, host genetic factors and environment factors.

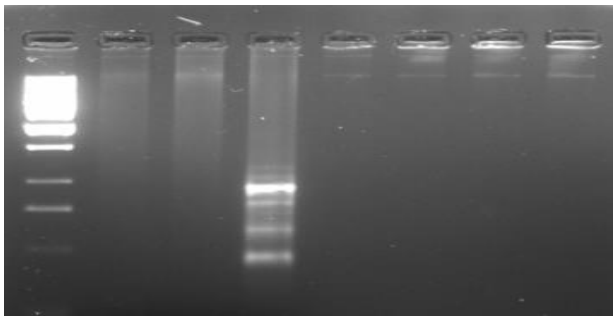


Fig. 6(a): PCR using random primer 1254. M is the 1kb ladder, lane 1-7 shows the RAPD-PCR amplicons.

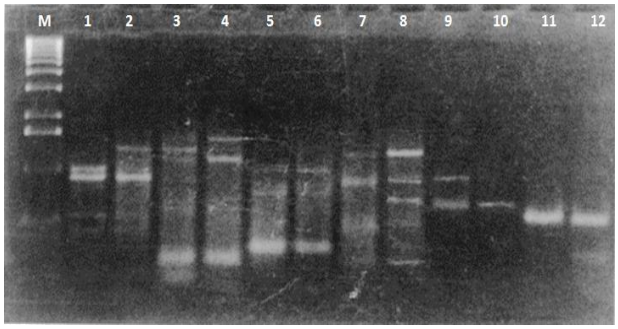


Fig 6(b): PCR using virulence gene 1283. M represents the 1kb ladder and lane 1-12 represents the RAPD PCR products showing polymorphism

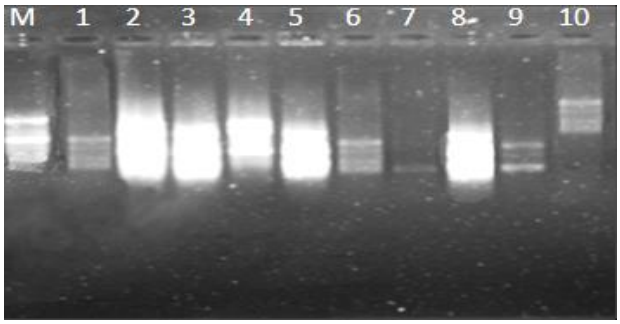


Fig 6(c): PCR using virulence gene cagA, iceA and vacA. M represents the 1kb ladder and lane 1-12 represents the RAPD PCR products showing polymorphism

Molecular marker assisted PCR based DNA fingerprinting has been valuable for studies of *H. pylori* genetic diversity and transmission.[22] The RAPD (random amplified polymorphic /arbitrarily primed (AP) technique has been widely used to obtain fingerprints of DNA and study genetic relatedness. Many studies have reported the involvement of certain *H. pylori* genotypes in more severe pathologies.[23] For the present study,

RAPD and multiplex PCR methods were used for the fingerprinting of isolated *H. pylori* strains. A total of 89 clinical isolates were obtained from the *H. pylori* positive patients (4-5 single colonies from each patient). These isolates scored positive in biochemical and molecular analysis. RAPD-PCR fingerprinting proved efficient as a preliminary determinant of genomic relatedness of the *H. pylori* isolates. Three infection types *i.e.* related, unrelated and mixed were identified based on random primers 1281, 1283 and 1254. Identical RAPD fingerprints of the isolates indicated no mixed-type infections. The results showed that primer 1283 was more sensitive compared to 1254 and 1281 in differentiating infections in our patients. Primer 1283 showed 37%, 24% and 18% sensitivity for three infection types, while it was 26%, 18% and 11% in case of primer 1254. Eleven patients (27%) showed similar RAPD profiles (Fig. 6a, b). RAPD technique if highly standardized (including careful DNA extraction and PCR assay) can prove efficient and reproducible.[24]

To determine evolution of their main virulence factors multiplex-PCR was individually performed for all isolates from each patient.[11] Analysis of the PCR results revealed frequencies of cagA, vacA, iceA as 84%, 47% and 33% respectively (Fig. 6c). 21 out of 40 patients showed the presence of multiple *H. pylori* strains in a single individual (51%). The preliminary results obtained in this study indicate that RAPD markers can be effectively used for preliminary genomic fingerprinting of various *H. pylori* strains.

Studies indicate that presence of various *H. pylori* genotypes may be a mode of adaptation employed by the bacteria for its survival in the host's ecological niches of the gastric mucosa creating a competitive environment for the resident strains.[25] Studies have reported heterogenous frequencies of cagA, vacA depending upon the geographical locations. For *e.g.* The frequency of *cag* A positive samples varies between (59-75)% in most Western European countries and the United States, while in Italy and Portugal, the rates are ≥80% and in Mexico, almost all the samples show colonization by *H. pylori* isolates harbouring the gene.[10,11,26] These results supplemented with detailed allelic discrimination of *H. pylori* genes could be highly beneficial for deciding treatment regimes. Research on larger sample size will generate better clinical and molecular profiles of host and pathogen. More focus should also be laid on the genetic variability of virulence genes of *H. pylori*.

ACKNOWLEDGEMENT

The authors acknowledge the support of The Coordinator, Bioinformatics lab, School of biotechnology, University of Jammu, Jammu for providing the internet facilities.

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Source of Support: Nil. **Conflict of Interest:** None Declared.

Cite this article as: Shelly Sehgal, Sachin Gupta, Sanjana Kaul, Manoj K Dhar. Genetic Diversity among Clinical Isolates of *Helicobacter Pylori* Detected From India. Int J Med Res Prof. 2017; 3(1):92-97. DOI:10.21276/ijmrp.2017.3.1.017