GENETIC VARIATIONS AND STATUS OF PATHOGENICITY GENES IN *Helicobacter pylori* IN PATIENTS OF NORTH AND SOUTH INDIA

THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

Under the Supervison of
Prof. Ashok Kumar

By
Sushil Kumar

School of Biotechnology
Banaras Hindu University
Varanasi-221 005
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Enrolment No. 258917 **2009**

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Dedicated to
My Grandfather
(Late)
and Parents

Contents

Preface

Helicobacter pylori is a pathogen that is believed to be a major risk factor in the development of duodenal ulcer and gastritis. Chronic gastritis caused by *H. pylori* infection, perhaps alongwith exposure to dietary and environmental mutagens, ultimately leads to gastric cancer. Living in a developing country and low socioeconomic living conditions for those in developed countries include some of the major environmental risks for infection. Moreover, most infections are apparently acquired in childhood. Once well established *H. pylori* is not eliminated by the host's normal immunological defence mechanism and probably persists life long in the most untreated patients.

H. pylori is a highly successful bacterial pathogen that persistently colonizes the mucosa of the human stomach and grows under such a harsh environment. There is increasing evidence that distinct variants of *H. pylori* exist and that these may be associated with the pathogenicity of the bacterium; several virulence-associated genes have been identified.

Eventhough, only a fraction of colonized individuals develop clinical sequelae which perhaps depend on factors like host characteristics that are governed by genetic polymorphisms, differentially represented bacterial determinants, and/or the specific interactions between a particular strain and its host that occur during decades of coexistence. Initially the *H. pylori* population structure appears to be clonal, substantial genetic diversity is seen in isolates obtained from different individuals that are consistent with extensive recombination and a panmictic population structure.

Helicobacter pylori infection is strongly associated with gastric cancer. The causes of gastric cancer are not well undestood, although nutritional and genetic factors have been suggested to be involved in a multifactorial and multistage process. El-Omar *et al*. (2000) reported the first positive association between gastric cancer risk and polymorphisms in the genes coding for the cytokines interleukin 1B (IL-1B) and interleukin 1 receptor antagonist (IL-1RN).

Genotypic variations in pathogen populations possess a major barrier to disease control. As of today, amongst the pathogenic microorganisms, diversity of bacterial pathogen is the most inetersting area of research. Gastrointestinal diseases are very

common among Indians. Unfortunately little, if any, work has been conducted on the prevalence and genetic variations among the most notorious pathogeneic bacterium, *H. pylori* especially from North India isolates. I have made an attempt to reveal certain facts related to the factors involved in pathogenecity and the role of host factors in appearance and development of gastric diseases caused by *H. pylori*.

I wish to express my heartfelt thanks and immense gratitude to my supervisor Prof. Ashok Kumar who introduced me to this research area and inculcated scientific temper in me. He always provided me his valuable suggestions, inspiration, encouragement, moral support and guidance for sound completion of research work. His continuous cooperation in sorting out my queries patiently will always be in my heart for life long. I am highly indebted to him for his inspiring and constructive guidance. My sense of appreciation to him cannot absolutely frame in the boundary of words.

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It is too hard to express feelings in words tor my wife Prabha who shared my emotions in my plesure and pain and consolidated my efforts through her love, sacrifice and support through out my research period.

At the last but not least, I pay my strong reverence to my parents without whose blessings my dream of present endeavor would never been fructified and whose boundless love, constant inspirations, endurance and sacrifice made me able to achieve this goal. I cannot forget the affection and moral support of my brothers Mr. Anil Kumar and Arvind Kumar, and my sisters and father/mother in laws for their affection cooperation, continual encouragement and everlasting blessings which helped me a lot in keeping my spirits up for achieving the goals.

Lastly, I am also grateful to University Grants Commission, New Delhi, India, for the financial support in the form of award of Junior Research Fellowship.

Sushil Kumar

Abbreviations

AFLP : Amplified Fragment Length Polymorphism

ARDRA : Amplified Ribosomal DNA Restriction Analysis

BLAST : Basic Local Alignment Search Tool

*cag*A : Cytotoxin Associated Gene A

DDBJ : DNA Database of Japan

DGGE : Denaturing Gradient Gel Electrophoresis

DU : Duodenal Ulcer

ELISA : Enzyme-Linked Immunosorbent Assay

EMBL : European Molecular Biology Laboratory

ERIC : Enterobacterial Repetitive Intergeneric Consensus

GERD : Gastroesophageal Reflux Disease

IL : Interleukin

IL-RN : IL-1 receptor antagonist

LPS : Lipopolysaccharide

MALT : Mucosa Associated Lymphoid Tissue

NAP : Neutrophil Activating Protein

NSAID : Non-Steroidal Anti-Inflammatory Drugs

OR : Odds Ratio

ORF : Open Reading Frame

PAI : Pathogenicity Island

PPI : Proton Pump Inhibitors

PU : Peptic Ulcer

RFLP : Restriction Fragment Length Polymorphism

RUT : Rapid Urease Test

UBT : Urea Breath Test

URUT : Ultra Rapid Urease Ttest

vacA : Vacuolating Cytotoxin A

Abstract

Helicobacter pylori is a highly successful bacterial pathogen that persistently colonizes the mucosa of the human stomach. The works described in this thesis include the role/involvement of environmental factors, occurrence of genetic variability and the role of host genetic factors in the pathogenesis of *H pylori*-induced gastric diseases particularly in North Indian population. Research works started in collaboration with Gastroenterology Dept., Institute of Medical Sciences, along with Sir Sundarlal Hospital, Banaras Hindu University, Varanasi. The study involved 276 cases and among them, the prevalence of *H. pylori* was detected in 81.9%. On the basis of survey it was noted that the prevalence of *H. pylori* increased with age, number of family members, sources of drinking water, dental habits like use of toothpaste etc. However its prevalence was negatively associated with the factors like smoking, intake of citrus fruit juices, exercise habits and schedule of taking food.

Studies related to pathogen factors were conducted by the development of a novel multiplex PCR meant for diagnosis as well as characterization of important pathogenicity genes such as *cagA* and *vacA* and 16S rRNA gene. The method adopted is based on the use of intact bacterial cell as template instead of purified/unpurified genomic DNA for amplification of desired amplicons. Out of 276 patients, 182 showed single *H. pylori* strain, 36 had multiple *H. pylori* strain and 8 patients although had single *H. pylori* strain but showed amplification of 16S rDNA only. Dominant *vacA* genotype was s1 and m1 being present in 92.3% and 58.2% patients respectively followed by m2, and the lowest being s2. Analysis of *cagA* gene revealed its presence in 63.2% cases. Detection of *H. pylori* seems rapid and simple by the method adopted by us. Additionally, genotyping of *vacA* and *cagA* genes could also be routinely performed in a large number of patients for diagnostic purposes using this method. In addition to above, molecular diversity analysis was conducted with thirty six isolates (14 Varanasi and 22 Hyderabad) by RFLP of 16S rRNA gene, ERIC–PCR and AFLP. All the isolates showed distinguishable banding pattern by AFLP. Dendrogram based on AFLP profile revealed that all these isolates could be placed in 3 major groups. Phylogenetic tree based on 184 partial 16S rDNA sequences (13 Varanasi, 11 Hyderabad and 160 reference strain) suggested that Indian isolates are closely related to the isolates of Brazil and/or Taiwan. We have also studied the diversity of *cag* PAI between North and South Indian populations with an aim to reveal the status and integrity of *cag*-PAI. Results obtained revealed significant differences between North and South Indian isolates.

The relationship between interleukin 1B (IL-1B) polymorphism, *H. pylori* infection, and gastric cancer (GC) in patients of North India was evaluated. The study included biopsy tissues of 136 cancer patients and 110 controls. Polymorphisms in IL-1B-511, -31, +3954 and IL-1RN genotypes and *H. pylori* active infection were analysed by PCR-RFLP. The frequency of IL-1RN 2/2 was significantly higher in GC cases than the controls. The risk of gastric cancer markedly increased in the genotypes of IL-1B -511 TT, -31CC, +3954 CT and IL-1RN 1/2 when infected with *H. pylori*.

Chapter 1

GENERAL INTRODUCTION

A brief history of *H. pylori* infection

About 25 years ago, Barry Marshall and Robin Warren described the successful isolation and culture of a spiral bacterial species from the human stomach (Warren and Marshall, 1983). During that period (1982-83) the conventional thinking was that no bacterium can live in the human stomach as the stomach produced excessive amounts of acid which was similar in strength to the acid found in a car-battery. The organism was initially named "*Campylobacter*-like organism," "gastric *Campylobacter*-like organism," "*Campylobacter pyloridis*," and "*Campylobacter pylori*" but is now named *Helicobacter pylori* in recognition of the fact that this organism is distinct from the members of the genus *Campylobacter* (Goodwin *et al.,* 1989). Self-ingestion of *H. pylori* experiments by Marshall *et al.,* (1985a) and Morris and Nicholson (1987) and later experiments with volunteers (Morris *et al.,* 1991), demonstrated that these bacteria can colonize the human stomach, thereby inducing inflammation of the gastric mucosa (Fig. 1.1). Marshall developed a transient gastritis after ingestion of *H. pylori*; the case described by Morris developed into a more persistent gastritis, which was resolved after sequential therapy, first with doxycycline and then bismuth subsalicylate. These initial data strongly stimulated further research, which showed that gastric colonization with *H. pylori* can lead to variety of upper gastrointestinal disorders, such as chronic gastritis, peptic ulcer disease, gastric mucosa associated lymphoid tissue (MALT) lymphoma, and gastric cancer. This knowledge had a major clinical impact with regard to the management of these diseases. In addition, the persistence of a pathogen in an environment long thought to be sterile also resulted in insights into the pathogenesis of chronic diseases. This discovery resulted in the award of the 2005 Nobel Prize in Physiology and Medicine to Robin Warren and Barry Marshall for their "discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease." Although the prevalence of *H. pylori* in the Western world is decreasing, gastric colonization by *H. pylori* remains widespread in the developing world. Infection with *H. pylori* can be diagnosed by a variety of tests and can often be successfully treated with antibiotics. Unfortunately, the increase in antibiotic resistance is hindering the efficacy of treatment, and, in spite of the pathogenicity of *H. pylori*, preventive vaccination strategies still do not exist. A better

understanding of *H. pylori* persistence and pathogenesis is thus mandatory to aid the development of novel intervention and prevention strategies.

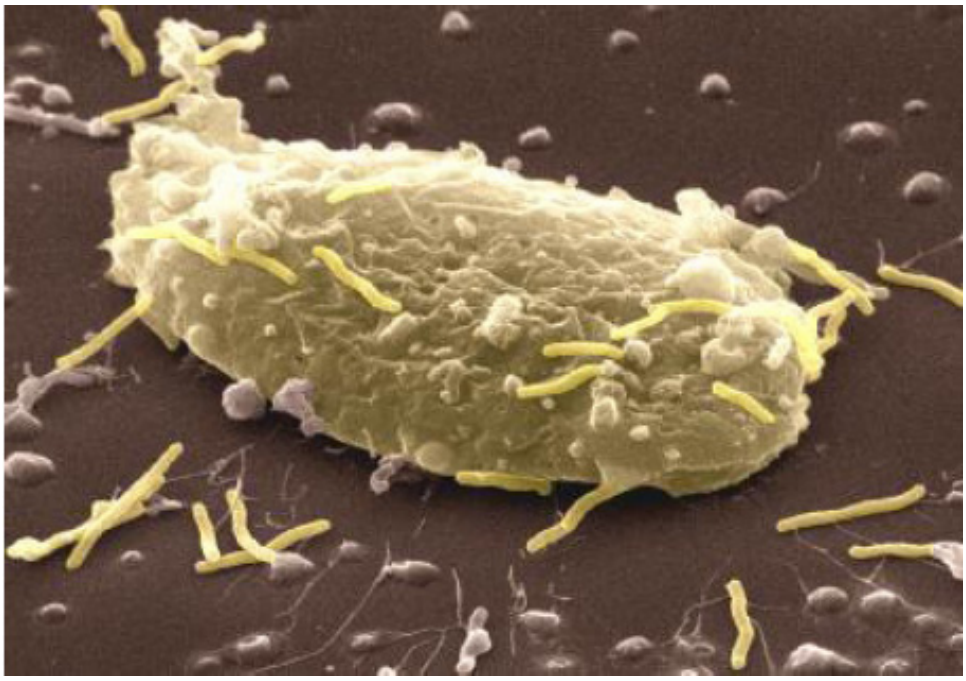

Fig. 1.1 Electron microscopy of *H. pylori* adhered with human epithelial cell culture of gastric tissue.

1 Microbiology

1.1 Genus description and phylogeny of *Helicobacter*

The genus *Helicobacter* belongs to the ε subdivision of the *Proteobacteria*, order *Campylobacterales*, family *Helicobacteraceae*. The most important stage in the development of the taxonomy was proposed by Goldwin and colleague (Goodwin *et al.*, 1989) to establish a new genus called *Helicobacter*. They proposed that *C. pylori* should be transferred to the genus *H. pylori*. The creation of the new genus was argued mainly on the basis of the 16S rRNA (small subunit) sequence data (Owen, 1995). To date, the genus *Helicobacter* consists of over 20 recognized species but many of them is awaiting formal recognition (Fox, 2002). Members of *Helicobacter* genus are all microaerophilic organisms and in most cases are catalase and oxidase positive, and many but not all species are also urease positive.

1.2 Bacteriology of *H. pylori*

1.2.1 Growth requirements

A wide range of basal media and supplement have been used for the primary isolation of *H. pylori* from gastric biopsies (Kusters *et al*., 2006). *H. pylori* is a fastidious microorganism and requires complex growth media. Solid media have been commonly used for routine isolation and culture consisting of Columbia, Brain Heart Infusion agar (BHI) or *Brucella* agar supplemented with either (lysed) horse or sheep blood or alternatively, newborn fetal calf serum. Most solid media used as a selective medium contain an antibiotic supplement such as vancomycin (10 mg/l), polymyxin B (2500 units/l) and trimethoprim (5 mg/l) Skirrow (1977) or cefsoludin (5 mg/l), vancomycin (10 mg/l), amphotericin B (5 mg/l) and trimethoprim (5 mg/l) Dent and McNulty (1998). *H. pylori* is microaerophilic, with optimal growth at O_2 levels of 2 to 5% and the additional need of 5 to 10% CO_2 and high humidity. Growth of *H. pylori* occurs at 34 to 40°C, with an optimum at 37°C. Colonies of *H. pylori* from primary culture on solid agar usually take 3 to 14 days to appear and are small (~1-mm), translucent smooth colonies (Han *et al*., 1995). Although its natural habitat is the acidic gastric mucosa, *H. pylori* is considered to be a neutrophile. The bacterium will survive brief exposure to pHs of <4, but growth occurs only at the relatively narrow pH range of 5.5 to 8.0, with optimal growth at neutral pH (Scott *et al*., 2002).

1.2.2 Morphology

H. pylori is genetically described as a gram-negative bacterium, measuring 2 to 4 µm in length and 0.5 to 1 µm in width. Although usually spiral-shaped or curved rod with one to three turns when observed *in vivo*, the coccoid shapes appear after prolonged *in vitro* culture or antibiotic treatment (Kusters *et al*., 1997). These coccoids loose their ability to be cultured *in vitro* and are thought to represent dead cells (Kusters *et al*., 1997), although it has been suggested that coccoid forms may represent a viable, non-culturable state (Enroth *et al*., 1999). The organism has 2 to 6 unipolar (lophotrichate), sheathed flagella of approximately 3µm in length, which often carry a distinctive bulb at the end of flagellum (O'Toole *et al*., 2000). Bacterial cells are mostly actively motile in viscous solutions such as the mucus layer overlying the gastric epithelial cells (O'Toole *et al*., 2000).

1.2.3 Genome, plasmids and strain diversity

The genome (chromosomal) DNA of *H. pylori* is a single circular molecule. The size of the five sequenced *H. pylori* (Shi 470, HPAG1, J99, 26695 and G27) genome is approximately 1.7 Mbp, with a G+C content of 35 to 40%. The *H. pylori* strain 26695 genome includes 1,587 genes, whereas the genome of strain J99 includes only 1,491 genes (Alm *et al.*, 1999; Tomb *et al.*, 1997). Genomes of J99 and 26695 contain two copies of the 16S, 23S, and 5S rRNA genes. Many strains (ca 45%) carry one or more cryptic plasmids, which do not seem to carry genes responsible for virulence or antibiotic resistance (Heuermann and Haas, 1995). Generally bacterial pathogens are highly clonal (such as *Shigella dysenteriae* and *Mycobacterium tuberculosis*), in contrast, *H. pylori* is genetically heterogeneous, suggesting a lack of clonality. This results in every *H. pylori*-positive subject carrying a genetically distinct strain (Kusters *et al.*, 2006), although differences within relatives may be small. The regions of genetic heterogeneity are possibly an adaptation of *H. pylori* to the harsh gastric conditions of its host, as well as to the distinct patterns of the host-mediated immune response to *H. pylori* infection (Kuipers *et al.*, 2000). Genetic diversity is thought to occur via several methods of DNA rearrangement and the introduction and deletion of foreign sequences (Achtman and Suerbaum, 2000; Falush *et al.*, 2003; Suerbaum and Achtman, 2004). The latter usually have an aberrant G+C content and often carry genes involved in virulence. A striking example of this is the cytotoxic associated gene, Pathogenicity Island (*cag* PAI), but other plasticity regions have also been suggested to play a major role in the pathogenesis of *H. pylori* infection (de Jonge *et al.*, 2004a; Santos *et al.*, 2003). At the molecular level, the diversity in genome may be seen via several mechanisms, including transcriptional and translational phase variation and mutation (Achtman and Suerbaum, 2000; Falush *et al.*, 2001; Suerbaum and Achtman, 1999). Often phase variation occurs via reversible slipped-strand mispairing in homopolymeric G or C tracts, resulting in a shift in translation of the affected gene, thus finally phase variation via a single mutation. This event shows a reversible phenotypic diversity with only minor genetic variation. Several virulence genes, namely *sabA*, *sabB*, *hopZ*, and *oipA* outer membrane protein-encoding genes, display such phenotypic variation, as do lipopolysaccharide (LPS) biosynthetic enzymes (Aspholm-Hurtig *et al.*, 2004; de Jonge *et al.*, 2004b; Kusters *et al.*, 2006; Mahdavi *et al.*, 2002).

2 Epidemiology

2.1 Prevalence and geographical distribution

The prevalence of *H. pylori* shows wide geographical variations in different parts of the world. More than 80% of the population of various developing countries are *H. pylori* positive (Kusters *et al*., 2006). In the industrialized countries, *H. pylori* prevalence generally remains under 40%. Prevalence of *H. pylori* is considerably lower in children and adolescents than in adults and elderly people (Pounder and Ng, 1995). Within particular geographical areas, the prevalence of *H. pylori* is inversely proportional with socioeconomic status, this is due to living standard during childhood (Malaty and Nyren, 2003). *H. pylori* infection rates rise rapidly in the first 5 years of life in developing countries and remain constantly high thereafter, indicating that *H. pylori* is acquired early in childhood (Fiedorek *et al*., 1991). However, *H. pylori* prevalence is low in early childhood and slowly rises with increasing age in industrialized countries. Overall, new *H. pylori* infection is more common in childhood and persists for life long unless specifically treated.

3 Diseases associated with *H. pylori* infection

3.1 Gastritis

It is well known that infection with *H. pylori* always causes chronic active gastritis. Gastritis commonly refers to inflammation of the lining of the stomach. Infection with the *H. pylori* provokes invasion of the mucosa by lymphocytes/plasma cells (a marker for the grade of gastritis), and neutrophils (a marker for the activity of gastritis). The association between gastrointestinal (GI) symptoms and chronic superficial gastritis (Marshall and Warren, 1984) is uncertain. However, it sometimes, develops into chronic atrophic gastritis with loss of epithelial glands over a period of decades. In children, *H. pylori* infection is associated with the development of chronic gastritis (Hatakeyama and Higashi, 2005). A large-scale investigation based on endoscopy of 396 Greek children with recurrent abdominal pain (RAP) revealed no significant differences in frequency and character of symptoms between *H. pylori* positive and negative groups (Roma *et al*., 1999). Gastritis was histologically confirmed to be the most prominent finding in *H. pylori* positive (98%) as compared to negative (19%) children ($p<0.001$) (Roma *et al*., 1999).

3.2 Peptic ulcer

Gastric or duodenal ulcers (peptic ulcer) is defined as a defect in the gastric mucosa of at least 0.5-cm in diameter and penetrating through the muscularis mucosa, with or without bleeding and perforation (Kuipers *et al*., 1995). Peptic ulcer (PU) disease is strongly associated to *H. pylori* infection. The discovery of *H. pylori* infection has dramatically changed the understanding of the pathogenesis of ulcer disease and also its treatment. *H. pylori* infection is diagnosed in 95% of duodenal ulcer (DU) and 85% of gastric ulcer occurred in the presence of the infection of *H. pylori* worldwide (Borody *et al*., 1991; Kuipers *et al*., 1995). The remaining DUs and PUs, especially in elderly, are due to drugs like NSAID use and other rare causes like Zollinger-Ellison syndrome and Crohn's disease (Borody *et al*., 1991). Occurrence of most ulcers is at sites where mucosal inflammation is most severe. The corresponding prevalence of infection in the case of gastric ulcer patients has been more complicated to establish since biopsy based diagnosis generally shows false-negative results due to association between gastric ulcers and atrophic changes of the mucosa. Serological confirmation of *H. pylori* infection has, however, verified an *H. pylori* seroprevalence of 60-100% in gastric ulcers (Kuipers *et al*., 1995). Seroepidemiological evidence from different populations revealed that the lifetime risk for peptic ulcer disease among *H. pylori* infected individuals is 6-20%, which is at least 3-4 times higher than for uninfected individuals (Feldman *et al*., 1998; Kuipers *et al*., 1995). Ulcer development in the presence of *H. pylori* is influenced by variety of host, bacterial and environmental factors.

3.3 Nonulcer dyspepsia

Dyspepsia is simply, a term which includes a group of symptoms that come from a problem in upper gastrointestinal tract. Non-ulcer or functional dyspepsia is defined as the presence of symptom of upper gastrointestinal distress without any identifiable structural abnormality during diagnostic work-up, in particular including upper gastrointestinal tract. The main symptom of dyspepsia is usually pain or discomfort in the upper abdomen, in addition, may be, heartburn, bloating, belching, quickly feeling 'full' after eating, feeling sick (nausea) or vomiting. Symptoms are often related to eating. 30 to 60% of patients with nonulcer dyspepsia carry *H. pylori*, but this prevalence is not much different from that in the unaffected population (Kusters *et al*., 2006). Two randomised double-blind placebo controlled studies in adults with nonulcer dyspepsia did not find

association between *H. pylori* eradication and relief of symptoms (Blum *et al*., 1998). However, a third well-designed trial reported a benefit of eradication treatment in a small subgroup of patients (McColl *et al*., 1998).

3.4 Gastric cancer

Gastric adenocarcinoma is the second most common cause of cancer related death world wide and 14th cause of over all death (Parkin *et al*., 1999). Gastric adenocarcinoma is histopathologically subdivided into intestinal type and diffuse type. Intestinal-type gastric adenocarcinoma, which occurs in older people, is more common than diffuse type. A small subset of diffuse type adenocarcinoma is of familial origin, caused by mutations in the *E-cadherin* gene (Guilford *et al*., 1998). Epidemiological studies as well as infection studies using animal models have indicated that *H. pylori* plays a critical but major role in the development of both types of gastric adenocarcinoma (Shimizu *et al*., 1999; Uemura *et al*., 2001). A large-scale prospective study also revealed that the risk of development of gastric carcinoma was much greater in the population infected by *H. pylori* than in the *H. pylori*-uninfected population (Uemura *et al*., 2001) (Fig. 1.2). Eradication of *H. pylori* significantly decreased the rate of gastric carcinoma development in *H. pylori*-infected patients without precancerous lesions, providing evidence for the causative role of *H. pylori* in gastric carcinogenesis (Wong *et al*., 2004). The bacterium colonizes over half of the population worldwide. Infection is usually acquired in childhood and in the absence of antibiotic therapy and therefore persists for the lifelong of the host. Although most infected patients will develop a chronic active gastritis, the majority are asymptotic. Though the risk varies with age, geographical location, diet and ethnicity, overall 15-20% of infected patients will develop gastric or duodenal ulcer disease and only a fraction of all *H. pylori* infected (1-2%) is likely to develop gastric carcinoma (Kuipers *et al*., 1998). A multitude of host and environmental factors, including interaction with other infectious agents and dietary factors, affect the immune response to *H. pylori* and may impact disease presentation over the life of the host (Stoicov, *et al*., 2004).

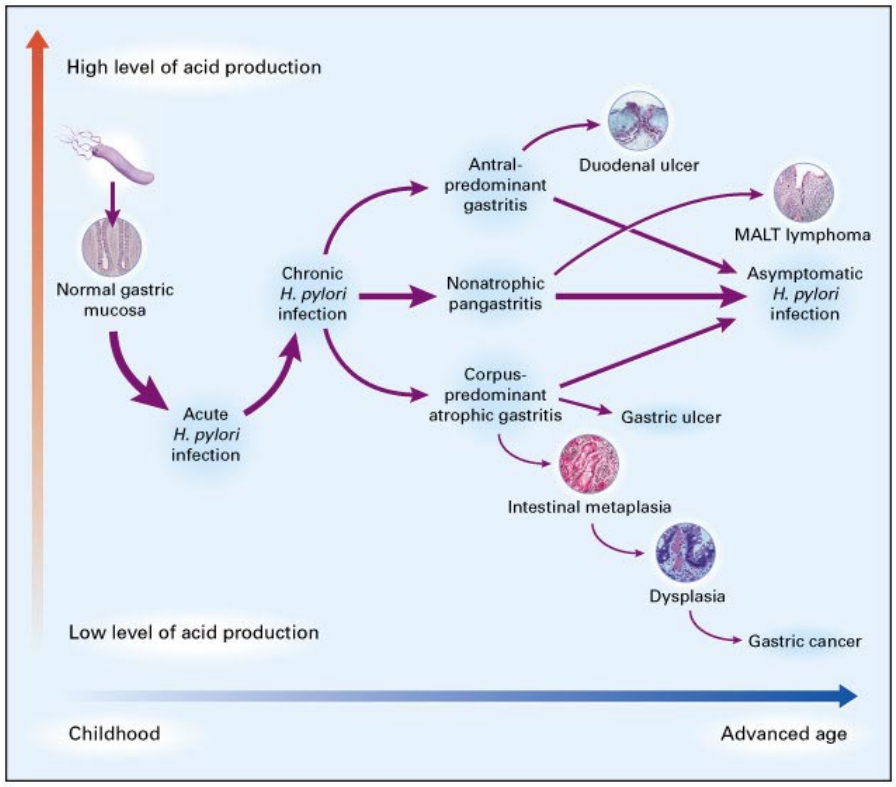

Fig. 1.2 *H. pylori* is usually acquired in childhood and develops acute infection leading to chronic gastritis in virtually all persistently colonized persons. Eighty to ninety percent infected persons never show any symptoms. Clinical outcome is highly variable and depends on bacterial and host factors. Higher acid secretor patients, due to infection and host genetic factor, are likely to develop antral-predominant gastritis, which leads to develop duodenal ulcers. Lower acid secretor patients are more likely to have gastritis in the body of the stomach, which leads to develop gastric ulcer and can initiate a sequence of events that, in rare cases, finally develop in gastric carcinoma (Uemura, *et al*., 2001).

3.5 Gastroesophageal reflux disease

Gastroesophageal reflux disease (GERD) is defined as chronic symptoms of mucosal damage produced by the abnormal reflux of gastric contents into the esophagus (Kenneth *et al*., 1999). Even though GERD is primarily a motility disorder, other pathophysiological disturbances seem to play a role in its pathogenesis. Spectrum of the esophagus injury includes esophagitis, stricture, the development of columnar metaplasia in place of the normal squamous epithelium (Barrett's esophagus) and adenocarcinoma (Kahrilas, 2008). GERD has long been considered to occur independently of *H. pylori* infection, i.e., to occur with the same frequency and severity in *H. pylori*-positive and *H. pylori* negative subjects (Kuster *et al*., 2006). However, further studies suggested that the

curing of *H. pylori* infection in DU patients may provoke reflux esophagitis (Labenz *et al*., 1997). A 2-year follow-up, based on 276 DU patients randomised to either eradication therapy or long-term omeprazole treatment, revealed that both regimens were equally effective in controlling dyspeptic symptoms and GERD in patients with a healed DU (Bytzer *et al*., 2000). Furthermore, the healing of ulcers was not found to increase the risk of GERD.

3.6 Extragastric manifestations

Till date there is a general belief that *H. pylori* is linked to a variety of extragastric diseases, thereby opening the new "extragastric manifestations of *H. pylori* infection" field (Hilde *et al*., 2008). These include coronary heart disease, dermatological disorder such as rosacea and idiopathic urticaria, autoimmune thyroid disease and thromophenomenon, scleroderma, migraine, iron deficiency anemia and Guillain-Barre syndrome (Kuster *et al*., 2006; Ulrich *et al*., 2007). Sidropenic anaemia has been reported to be associated with *H. pylori* infection and the reversal of iron deficiency anaemia after eradication of *H. pylori* has been observed in many patients (Kuster *et al*., 2006). The observations might be due to subclinical bleedings from the GI tract but are also likely to be due to the genetic factors indicating that *H. pylori* scavenge iron, presumably from its host (Tomb *et al*., 1997). In some paediatric studies, association of *H. pylori* infection with short stature has been suggested (Goodman *et al*., 1997). However, confirmation of these observations would need further studies taking into account various factors such as genetic and socio-economic/nutritional conditions. Among children in developing country, a positive association has been noted between *H. pylori* and *Vibrio cholerae* (Shahinian *et al*., 2000). A higher *H. pylori* prevalence in children with chronic diarrhoea has been observed (Sullivan *et al*., 1990). In contrast, a population-based study of German pre-school children found an inverse association between *H. pylori* and diarrhoea (Rothenbacher *et al*., 2000).

4 Diagnoses

Invasive and non-invasive diagnostic methods

The invasive tests for *H. pylori* infections are based on gastric specimen for culture, histology, rapid urease assays and polymerase chain reaction (PCR) which by amplifying segments of DNA from *H. pylori* can detect very small numbers of bacteria

and characterize the various genes and this being direct methods. Since these methods require upper endoscopy, they are generally not considered suitable for paediatric epidemiological investigations, for ethical reasons and for the cost. The non-invasive methods based on peripheral samples such as blood, breath samples, stools, urine or saliva for detection of antibodies, bacterial antigens or urease activity all being indirect methods are preferred. The choice of a specific test for an individual patient depends on severity of disease, local experience, clinical setting and cost. In hospital-based care, many patients undergo endoscopy, which is then combined with an invasive test of *H. pylori.*

4.1 Culture

Culturing of bacterial colonies from gastric biopsies is regarded as a definitive proof of *H. pylori* infection. However, *H. pylori* is fastidious and microaerophilic organism, its culture is not always successful in many laboratory. The ability to culture the organism, and thus the sensitivity of the test, may vary between laboratories since the method is most technically demanding. The organisms are sensitive to temperature, oxygen and medium which create problem during transportation and the culture itself requires special conditions and 3-7 days of incubation. The main advantages of culture are the excellent specificity and the opportunity to test for susceptibility to antibiotics in patient management (Megraud, 1996). In addition, the genotype of the isolate with regard to *cag*A and *vac*A and other pathogenic gene status can be determined, although this investigation is limited to selected research centres. Genetic diversity study can also be easily made by culture of bacteria.

4.2 Histology

H. pylori is one of the microorganisms that can be confidently recognised by histology. Also, the histopathologists are uniquely placed both to identify infection and to assess the disease process associated with it, i.e., gastritis and in adults premalignant (dysplastic) changes or neoplastic lesions such as carcinoma, lymphoma or carcinoid. Even though the sensitivity of histology is generally high, this method may be affected by observer related factors and topographical changes in the stomach may introduce sampling errors. Although a single biopsy from the lesser curvature (2–3 cm from the pylorus) will yield a positive result in over 90% of infected stomachs (Genta and Graham, 1994), sensitivity can be improved by examining additional samples from the antrum and

corpus. Poor orientation or small biopsy samples will lower sensitivity. The most important factor affecting sensitivity is the experience and consciousness of the observer. Specificity is generally not a big problem. Difficulties arise when patients use anti-*Helicobacter* treatment where the bacterial count may be much reduced but infection not completely eradicated. If *H. pylori* adopts a coccoid form then confident identification becomes impossible. In this situation immunohistochemical staining (Cartun *et al*., 1990) and other technique such as *in situ* hybridization, (Bashir *et al*., 1994) or PCR (Kumar *et al*., 2008) may resolve the issue.

4.3 Rapid urease test

This test uses the high concentration of pre-formed urease enzyme activity in *H. pylori* infected gastric biopsy samples. The increasing alkalinity resulting from the production of ammonium ions ($NH4^{+}$) is detected by a colour change in the pH indicator and urea containing medium. The test can be carried out using small quantities of standard urea broth for in-house methods but commercial kits are also available. The sensitivity of the test depends on the number of bacteria present in the sample. It has been calculated that 10^4 microbes are required for a positive result, and a proportion of patients will harbor densities lower than this threshold (Megraud *et al*., 1996). Low levels of colonisation are to be expected if patients used anti-*helicobacter* treatment where the bacterial count may be much reduced but infection not completely eradicated. Increased sensitivity can be achieved by using two biopsy specimens and incubation at 37°C. The specificity of this rapid urease test is very good when the test is read at one hour, but declines with the length of the incubation. Thus, at 24 hours false positive results may be obtained by other urease producing bacteria (Moayyedi and Dixon, 1998). Recently many urease positive microbes other than *H. pylori* in human gastric juice and mucosa have been discovered (Brandi *et al*., 2006). In this situation this test will give false positive for *H. pylori*.

4.4 ^{13}C-Urea breath test

The urea breath test (UBT) is one of the most important non-invasive methods for detecting *H. pylori* infection. The test exploits the rapid hydrolysis of orally administered labelled urea by the enzyme urease, which is produced by *H pylori* in large quantities. Urea is hydrolysed to ammonia and carbon dioxide, which diffuses into the blood through mucus layer of the gastric mucosa via the systemic circulation and is excreted in the

expired air through lungs. ^{13}C-labeled urea is becoming increasingly popular because this non-radioactive isotope is innocuous and can be safely used in children and pregnant women. The test gives a direct measure of an active gastric infection (Logan, 1998), also reflecting the bacterial load and grade of histological gastritis (Debongnie *et al*., 1991). The test is easy to perform and can be repeated as often as required in the same patient. The UBT is a very sensitive and specific method, thus providing a gold standard against which the accuracy of other diagnostic tests (both invasive and non-invasive) can be validated (Hawtin, 1999). However, it may not be reliable in assessing patients who have had gastric surgery. One of the most important advantages of the UBT is that it samples the whole stomach and there is no chance of sampling error unlike biopsy based tests (invasive test), which can be influenced by the patchy distribution of *H. pylori* infection within the gastric mucosa. Occasionally, oral urease producing bacteria may cause false positive results (Hamlet *et al*., 1995). Additionally, many urease positive microbes other than *H. pylori* present in human gastric juice and mucosa may give false results (Brandi *et al*., 2006). Also, antibiotics, bismuth salts and PPI, which reduce the extent of *H. pylori* infection, may introduce false negative results. The cost of producing ^{13}C-urea is high, but it may be possible to reduce the dosage further by administering it in capsule. Another major disadvantage is the high initial cost for the test, caused by the need for expensive mass spectrometric equipment. Recommendation of UBT is for monitoring the effect of eradication treatments and it is also a suitable test for epidemiological study, particularly in children. However, reservations have been made since the test is less reliable in children <5 years and it is not yet evaluated among the youngest (<2 years) (Drumm *et al*., 2000).

4.5 Serology

Serological test is also one of the non-invasive tests. These tests are used for detection of *H. pylori* infection and have been helpful in epidemiological studies of prevalence, allowing for the development of preventive infection early in life (Leal *et al*., 2008). Serology is the easiest way to identify *H. pylori* passive infection in individuals not undergoing gastroendoscopy and this test is based on the principle of detecting the circulating antibodies against *H. pylori*. Common designs are based on antibody detection tests which include the Western Blot (WB) and enzyme-linked immunosorbent assay (ELISA) technique. The ELISA format has the advantage that many serum samples can be tested in parallel and the process can be completely automated (Leal *et al*., 2008). This

is the most widely used method due to its simplicity and cost effective features, speed, and minimal patient discomfort thereby making it suitable also for large-scale screening (Laheij *et al*., 1998). Advantage of this test is that the result is not affected by sampling errors like other invasive or non-invasive test. One of the major drawbacks of this test is that a positive test does not necessarily indicate a current (active) infection however similar to the UBT, the test is not affected by sampling errors. It has been demonstrated that initially lower specificity of the method was due to false positive results mainly due to nonspecific cross-reaction with other microorganisms, such as *Campylobacter* sp. (Perez-Perez *et al*., 2004). Many different types of antigen preparations can be used in the ELISA and the upper limit of normal values, i.e., the cut-off level, needs to be evaluated for each assay system and in the population being investigated. Although ELISA has proven to be highly accurate for the diagnosis of *H. pylori* infection in adults, in children it has shown variable sensitivity and specificity (Dzierzanowska-Fangrat *et al*., 2006; Jones *et al*., 2005). It has been suggested that the cut-off value needs to be corrected specially in the case of children since they produce lower antibody responses than adults (Andersen *et al*., 1994), not only due to delayed and low antibody production, but also due to the occurrence of maternal antibodies up to the age of 3-7 months (Gold *et al*., 1997). It has shown limited sensitivity (80%) and specificity (70%) of *H. pylori*-specific IgA and IgG antibodies detection in saliva from general population (Sonmezoglu *et al*., 2005), however, *H. pylori*-specific IgG in urine has shown more sensitivity (95.5%) and specificity (83%) than saliva based assay (Leodolter *et al*., 2003; Sonmezoglu *et al*., 2005). Most IgG diagnostic tests are serum-based.

Molecular methods

4.6 Polymerase chain reaction (PCR)

4.6.1 Standard PCR assay for the detection of *H. pylori*

The PCR was developed in 1983 by Kerry Mullis and therefore was quickly applied to the detection of *H. pylori*. This method revolutionized the study of DNA, especially after the introduction of a thermostable DNA polymerase obtained from *Thermophilus aquaticus* (*Taq* polymerase). Its application in the field of *H. pylori* concerns not only the detection of the bacterium but also its sequencing, quantification and detection of specific genes relevant to pathogenesis such as *cagA, vacA, iceA, babA*

etc, as well as in diversity study such as RAPD, AFLP, ERIC-PCR etc and specific mutations associated with antimicrobial resistance. Target choice is most important in the primers designing which must be specific for *H. pylori* but conserved in all strains of the species. Therefore, it is necessary to know the DNA sequence of the target in as many strains of *H. pylori* and as many strains of other bacterial species as possible. In the case of direct detection of *H. pylori* from specimen it is also necessary to know host DNA sequence. The first target of *H. pylori* gene used was the genes of the urease operon: *ureA* (Clayton *et al*., 1991) and *glmM*, formerly named *ureC* (De Reuse *et al*., 1997), and the 16S rRNA gene (Ho *et al*., 1991; Hoshina *et al*., 1990). 16S rRNA gene is highly conserved in bacteria and exhibits sequences which are specific for different species. Among the drawbacks of PCR are; (i) existence of possible *Taq* and other themostable polymerase inhibitors which can decrease the sensitivity of the reaction, and (ii) the specimen could be contaminated by exogenous *H. pylori* DNA or organism, which alters the specificity. To date several methods have been proposed to improve sensitivity and specificity of PCR. To improve DNA extraction, phenol extraction, CTAB miniprep method or the use of many commercial kits for DNA extraction and purification to eliminate the possible PCR inhibitors in target DNA have been employed (Wang *et al*., 1993). Recently, it has been demonstrated that use of an internal control helps in (Thoreson *et al*., 1999) the test of possible PCR inhibitors, it is also advantageous in checking false negative due to PCR inhibitor. If the target is too low, use of a nested or seminested PCR was suggested to escape false positive as well as false negative result (Kusters *et al*., 2006). However, the use of nested or heminested PCR increases the risk of airborne contamination by amplicons and must be discouraged. Methods such as the reverse dot blot line probe assay (LiPA) (van Doorn *et al*., 1998b) and liquid phase (DNA-enzyme immunoassay) (Lage *et al*., 1996; Monteiro *et al*., 1997a) have been proposed. Several authors have reported the use of reverse transcription-PCR (RT-PCR) assay (el-Zaatari *et at*., 1995; Engstrand *et al*., 1992; Oksanen *et al*., 1999; Peek *et al*., 1995). This method is based on mRNA, it determines the viability of the bacteria present, but no improvement in sensitivity has been shown in this method. A significant improvement in sensitivity of PCR was obtained with the introduction of real-time PCR method. As is always the case when a new method is more sensitive than the reference method, it is necessary to prove that the results of new methods are not giving false positive results. The specificity of PCR is not usually a problem when dealing with gastric biopsy specimens. However, the possibility of false positives arises if grinding

apparatus in the lab, endoscopes (Roosendaal *et al*., 1994) or biopsy forceps are not carefully cleaned.

4.6.2 PCR assay for the detection of pathogenicity genes of *H. pylori*

An important application of standard PCR is the genotyping and detection of specific pathogenic factors of *H. pylori*. There are two most important pathogenic factors i.e., the *cag* PAI and the polymorphism of the *vacA* gene. Other genes involved in pathogenicity (*oipA*, *dupA*, *iceA*) and adherence (*babA2*, *sabA*) can also be detected by PCR. When both *cagA* and *vacA* detection is performed, a strong association is seen between the presence of *cagA* and *vacA* s1, corresponding to strains with the highest production of cytotoxin (Audibert *et al*., 2000; Han *et al*., 1999; Hennig *et al*., 1999; Rudi *et al*., 1999; van Doorn *et al*., 1998a; Yamaoka e*t al*., 1998b) as well as more severe pathology and disease. Usefulness of PCR for detection of *H. pylori* in different types of biological products for invasive as well noninvasive tests, such as gastric juice, saliva, dental plaque and faeces has been emphasized (Bindayna *et al*., 2006; Bravos and Gilman, 2000; Shimada *et al*., 2001). Although sensitivity and specificity of this test has high practical consideration but due to cost it has limited use (Fennerty *et al*., 1998). Some of the investigator developed strip based detection by which *H. pylori* genes are amplified with a biotin-labelled PCR primer and subsequently analyzed by a single-step reverse hybridization on the strip (van Doorn *et al*., 1998b). However, other recently developed method especially, novel multiplex PCR assay seems very useful. This method does not require cultivation of bacteria, extraction of genomic DNA, boiling of biopsy samples or frequent steps of centrifugation. Using this assay the prevalence of type I and/ or type II strains, *vacA* signal sequence (s1 and s2) and mid-region (m1 and m2) alleles as well as the presence or absence of a *cagA* gene can be detected in a single reaction, directly from gastric biopsy samples. This method is cost effective, simple and fast (Kumar *et al*., 2008).

4.6.3 Real-time PCR

More recently, the development of real-time PCR has facilitated more rapid, specific and quantitative estimation of gene targets as they are amplified in real-time (Heid *et al*., 1996). Advantage of this technique is not only detection of *H. pylori* in a

quick and precise manner but also its quantification and the detection of point mutations associated with antibiotic resistance. The principle of this method is quantification of the amplicons formed in real time. This has been done by using an intercalating agent, e.g., SYBR green I dye, or the fluorescence resonance energy transfer (FRET) principle (Wittwer *et al*., 1997).

4.6.4 PCR for quantification

PCR is also used for quantitation of *H. pylori* initially by using a competitive PCR assay, followed by the development of a real-time PCR which has considerably facilitated the process. However, the exponential nature of DNA amplification is prone to burden the experimental data with significant standard error due to the tube-to-tube inherent variations also known as tube effect in amplification efficiency. Therefore it necessitates the introduction of a variable amount of internal standard (PCR mimic) of different sizes for the reliable quantitation of DNA or RNA templates by PCR and co-amplification of a constant amount of target DNA sequence with the same set of primers. A visual comparison allows the detection of bands with the same intensity indicating the amount of target DNA present (Monteiro *et al*., 1997b), however, a detection by colorimetric method is also possible (Ozpolat *et al*., 2000). The standard curve is constructed on the basis of band intensity as well as colorimetric data of internal standard templates (ordinate) against the log of the ratios of the PCR products amplified from the known amounts of wild-type template and standards (abscissa). The amount of unknown amount of wild template can be calculated on standard curve. The validation of this method i.e., quantity of *H. pylori* in gastric mucus correlated with other invasive tests as well as with the urea breath test (UBT) in a study made by Furuta *et al*. (1996). However, they also used this method for post-eradicated patient follow-up with a high predictive value (Furuta *et al*., 1998).

5 *H. pylori* infection parameters

Several different characteristics and products of *H. pylori* have been described as virulence factors. Infection scenario of *H. pylori* is very important to distinguish between colonization and virulence factors. The colonization factors are those that are necessary for the survival of *H. pylori* in the human stomach at the very first step/acute infection

when the *H. pylori* initially enters the stomach. The virulence factors are those, which aid in persistence of the infection of *H. pylori* such as toxins and factors causing tissue inflammation in the host.

5.1 *H. pylori* colonization factors

5.1.1 Urease

The first and most crucial requirement for the pathogen such as *H. pylori* is to enter the stomach and survives in the acidic milieu. To colonize the stomach, *H. pylori* is equipped with the urease enzyme and expression of this enzyme is a universal feature among all *H. pylori* strains. This enzyme is a 550 kDa protein complex, the cytosolic urease enzyme hydrolyses urea into ammonia and carbon dioxide, which neutralizes gastric acidity (Kusters *et al*., 2006). The ammonia increases the pH in periplasmic space and protects the bacteria from the acidic environment. *H. pylori* is well equipped to survive in strong acid but it is not an acidophile and needs to leave the gastric lumen, also to avoid discharge of the urease in the intestine gastric epithelium as it needs neutral pH environment. Activity of urease enzyme is tightly controlled by a pH-gated urea channel, UreI, which is open at low pH and closed at neutral pH conditions (Weeks *et al*., 2000), allowing the bacterium a precise level of control over its pH environment. A urease mutant was shown to be deficient in colonizing gnotobiotic piglets as compared to the wild type (Eaton *et al*., 1991) emphasizing the importance of this enzyme for survival and successful colonization.

5.1.2 Flagella

Motility is also important for colonization, to move through the viscous gastric mucus layer. *H. pylori* uses the polar flagella so that it can encounter the surface of the gastric epithelium. *H. pylori* has a unipolar set of 4-6 flagella which makes this bacterium highly motile (Suerbaum, 1995). These consist of two structural subunits: a major 53 kDa FlaA and a minor 54 kDa FlaB encoded by genes located at the *H. pylori* chromosome (Suerbaum *et a*l., 1993). Movement of flagella is guided by chemotactic factors, which include bicarbonate ions and urea (Yoshiyama and Nakazawa, 2000). *H. pylori* has been shown to possess the enzymatic ability to disrupt the oligomeric structure of mucin (Windle *et al*., 2000), which may help the pathogen to move freely in the mucus layer. Most probably protection of the flagella from acid-induced damage is by the flagellar

sheath, which is an extension of the outer membrane (Geis *et al*., 1993). Ottemann and Lowenthal (2002) showed that motility of bacterium is required for colonization of *H. pylori* in gastric mucosa of mice. It has been demonstrated in mouse animal model that mutant *H. pylori* strain which has non-motile flagella was less virulent in comparison to its wild type strain in the infectious dose. Motility is not only essential for bacteria to escape the acidic surrounding and reach the gastric mucus, but is also important for persistence of the *H. pylori* infection. The mucus layer always secretes mucus toward the lumen for protecting gastric epithelium from acid since motility is required for movement of the bacteria into the newly formed mucus.

5.1.3 Adhesion

A final step in colonization of the stomach is the adhesion of *H. pylori* to the gastric surface epithelium. It is prerequisite that the bacteria strongly adhere to the gastric tissue to accomplish colonization against the peristalsis movement of the gastric wall combined with the fast turn over rate of the epithelial cells. Adhesion of the bacteria to the epithelial layer of host is mediated by a large family of 32 related outer membrane proteins (Hop proteins) that include the adhesions (Alm *et al*., 1999). BabA is a 75-kDa adhesion protein molecule that mediates the attachment of *H. pylori* to Lewis b (α-1, 3/4-difucosylated) blood group antigens on human gastric epithelial cells (Boren *et al*., 1993; Falk *et al*., 1995). Three *bab* alleles namely *babA1*, *babA2*, and *babB* have been identified (Ilver *et al*., 1998). However, *babA1* and *babA2* are identical alleles except that *babA1* has a 10-bp deletion of the signal peptide sequence that leads to the elimination of the translational initiation codon. The *babA2* and *babB* alleles, which encode homologous proteins, have polymorphic midregion sequences but have conserved sequences in the 5' and 3' regions (Kusters *et al*., 2006; Ilver *et al*., 1998). Only the *babA2* gene product is necessary for Lewis b binding activity (Ilver *et al*., 1998). Bacterial strains which possess the *babA*2 gene adhere more tightly to epithelial cells and promote a more aggressive phenotype. It has been reported that BabA2 is associated with a higher incidence of gastric adenocarcinoma. One of the most common epitopes on the surface of gastro-intestinal epithelium and expressed in soluble glycoconjugates in saliva and tears of 80% of the population is the histo-blood group antigen Lewis b. The relation between development of gastrointestinal disease and the blood groups have been demonstrated half a century ago, where it has been reported that the incidence of peptic ulcer is higher among individuals of blood group 'O' phenotype. Moreover, the frequency of incidence

of peptic ulcer is more in Lewis b antigen secretor than non-secretor individuals. Based on the conditions of the host tissue, *H. pylori* can bind to a variety of different receptors on the host tissue and possibly adopts the properties of adhesin. When *H. pylori* colonizing the mucus, approaches the epithelial lining and adheres to the Lewis b antigen on the surface of the epithelial cells, adhesion and interaction with host cells induce inflammation and finally sialylation of tissue cells takes place. The bacteria also adapt to the new environmental conditions in the tissue and express a new adhesin allowing it to bind to sialylated structures for further enhanced adhesion (Mahdavi *et al*., 2002). Such precise and programmed adaptations increase the "phenotypic fitness" of *H. pylori* towards successful persistent infection.

5.2 *H. pylori* virulence factors

Several pathogenic factors contribute to the outcome of disease, but the most important ones are the virulence factors produced by any given strain. On the basis of the *cagA* and *vacA* genotypes, two types of *H. pylori* have been defined. Type I strains contain cytotoxin-associated gene A, *cag*A, and express a functional vacuolating cytoxin A, *vac*A, and these genes are associated with chronic gastritis, peptic ulcers and gastric carcinoma. Type II strains do not contain the cagA gene, and do not produce functional VacA cytotoxin. Type II strains are generally not associated with gastric pathology (Kumar *et al*., 2008).

5.2.1 Cytotoxin associated gene A (*cagA*)

CagA, a 120–145-kDa protein with a carboxy-terminal variable region (Cover *et al*,. 1990; Hatakeyama and Higashi, 2005) is the most important pathogenic factor of *H. pylori* that was first described as a virulence factor associated with peptic ulcers (Cover *et al*., 1990; Crabtree *et al*,. 1991). The *cagA* gene that encodes CagA is localized at one end of the *cag* pathogenicity island (*cag* PAI), (Censini *et al*., 1996), a 40 kb DNA segment containing 31 putative genes including *cag*A that was most likely incorporated into the *H. pylori* genome by a process of horizontal transfer. The *cag* PAI DNA segment revealed by comparison with similar genes in other bacteria, those encoding components of a bacterial type IV secretion system, is a molecular syringe through which macromolecules are delivered from inside of the bacteria to the eukaryotic cells. Clinically, infection with the *cagA*-positive *H. pylori* strains are associated with higher grades of gastric inflammation and severe atrophic gastritis and has been suggested to play an important

role in the development of gastric carcinoma than the *cagA*-negative strains (Hatakeyama, 2004).

Depending on the presence of virulence genes of the type IV secretion system translocation of CagA into host cells occurs, which lie in the *cag* PAI of *H. pylori*. Once injected into the gastric epithelial cells by the type IV secretion system, CagA localizes to the inner surface of plasma membrane and undergoes tyrosine phosphorylation by several members of the SRC family of kinases (SFK) such as SRC, FYN, LYN and YES (Selbach, *et al*., 2002; Stein, *et al*., 2002). Tyrosine phosphorylation of EPIYA motif of CagA by SFK occurs in the absence of any stimuli, the resulting SFK are constitutively activated in gastric epithelial cells. The above interaction indicates that the CagA might disrupt key signal-transduction pathways, contributing to cell transformation. Tyrosine phosphorylation of CagA occurs at a unique 5-amino-acid sequence- Glu-Pro-Ile-Tyr-Alu (EPIYA) motif that is present in multiple numbers in the carboxy-terminal variable region of the protein (Higashi, *et al*., 2002ab). On the basis of flanking sequence of EPIYA motif, EPIYA motif is part of four distinct EPIYA sites i.e., EPIYA-A, B C and D, each contains a single EPIYA motif which surrounds the amino-acid sequence. The CagA proteins from *H. pylori* strains of Western counties (Western CagA) such as Europe, North America and Australia, possess EPIYA-A and EPIYA-B sites, followed by the EPIYA-C site ('A-B-C' type EPIYA), which is placed within a 32, 40 and 34 amino acids respectively. This EPIYA-C segment, a 34 amino acid stretch is mostly repeated one to three times in different Western CagA species as a result of homozygous recombination or misaligned replication of a 102 bp *cag*A gene segment. The tyrosine residue of EPIYA-C segment is the major site of tyrosine phosphorylation by SFK in the gastric epithelial cells in Western CagA, whereas those present within the EPIYA-A and EPIYA-B segment are also phosphorylated at tyrosine residues but much less frequently than EPIYA-C segment (Higashi, *et al*., 2002b; Stein, *et al*., 2002). CagA proteins of *H. pylori* strains isolated in East-Asian countries such as Japan, Korea and China are known as 'East-Asian CagA', and possess the EPIYA-A, EPIYA–B and EPIYA-D site ('A-B-D' type EPIYA) but not repeatable EPIYA-C segment. The EPIYA-D site within the EPIYA-D segment of East-Asian CagA represents the major tyrosine phosphorylation site (Fig. 1.3). Once tyrosine phosphorylation by SFK takes place, CagA acquires the ability to bind specially to cytoplasmic protein (SRC homology 2) SHP-2 domain containing protein tyrosine phosphatase (PTP) called SHP-2. SHP-2 has two tandemly repeated SH2

domain termed as N-SH2 and C-SH2 protein tyrosine phosphatase domain. The physical complex formed between SHP-2 and CagA is detectable in cell infected with CagA positive *H. pylori in vitro* as well as *in vivo*. The CagA-activated SHP-2 potentiates ERK MAP kinase activity and regulates both cell morphology and cell growth. SHP-2 also dephosphorylates a cellular substrate and decreases the level of tyrosine phosphorylated cortactine, which is involved in the induction of hummingbird phenotype that is associated with elevated cell mortality.

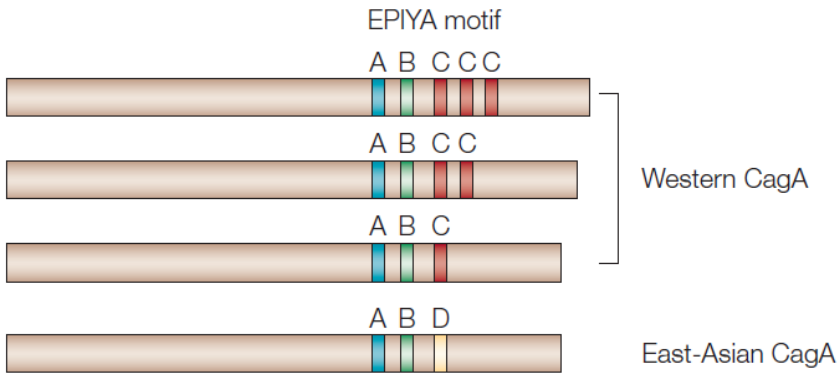

Fig. 1.3 Diversity in the tyrosine phosphorylation sites of CagA. Western strains of *H. pylori* express a form of CagA that contains the EPIYA-A, EPIYA-B and 1–3 repeats of EPIYA-C sites. East-Asian strains of *H. pylori* express a form of CagA in which the EPIYA-C site is replaced with the EPIYA-D site (From Hatakeyama, 2005).

5.2.2 Vacuolating cytotoxin A (*vacA*)

Vacuolating cytotoxin, VacA is a major virulence factor of *H. pylori* and causes cytoplasmic vacuolization in gastric epithelial cells (Leunk *et al*., 1988). This protein plays an important role in the pathogenesis of both peptic ulceration and gastric cancer (Kusters *et al*., 2006). The effect of the toxin was first observed on monolayer cell culture of eukaryote when vacuolization was induced by a cell-free supernatant of *H. pylori* culture (Leunk *et al*., 1988). The activities of VacA toxin include membrane channel formation, effects on integrine receptor-induced cell signalling, disruption of endosomal and lysosomal activity, interference with cytoskeleton dependent cell function, induction of apoptosis and immune modulation (Kusters *et al*., 2006). A chromosomal gene of *H. pylori* encodes the VacA responsible for the above effects. The VacA protein is produced as a 140 kDa protoxin that is cleaved into the 88-kDa mature form when secreted into the

extracellular space as soluble protein, but can also remain localized on the surface of *H. pylori* (Cover *et al*., 2005) (Fig. 1.4A). Although nearly all *H. pylori* secrete VacA toxin, there is considerable variation in vacolating activities among strains (Fig 1.5A). This is due to the sequence variability within the signal region (s) and the middle region (m) of *vacA* gene. The N terminal known as s of the gene encodes the signal peptide, and occurs as either an s1 or s2 type, whereas the C terminal known as m, which contains the p58 cell binding domain, exists as an m1 or m2 type (Fig. 1.4B).

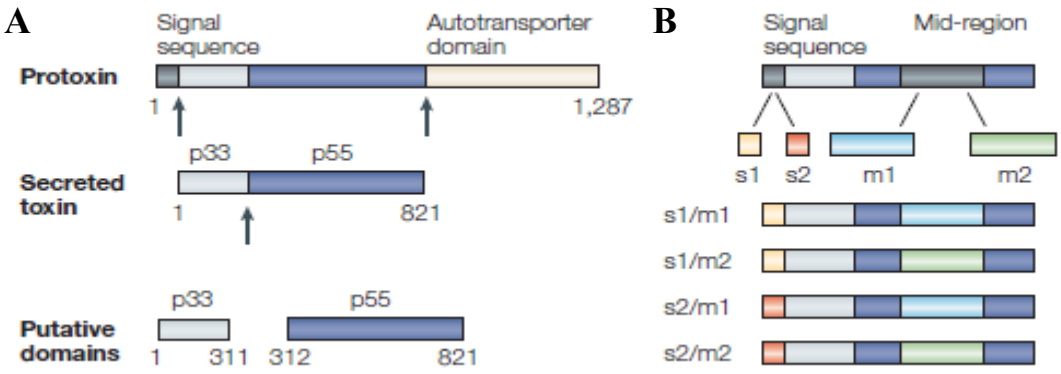

Fig. 1.4AB *vacA* gene structure and allelic diversity. **A-** 140-kDa VacA protoxin is cleaved to 88-kDa mature toxin that is secreted into the extracelluar space. Arrows indicate sites of proteolytic cleavage. This protein is able to mediate its own secretion. It is predicted that a 33-kDa C-terminal β-barrel domain of VacA is inserted into the outer membrane that forms a channel, through which secretion of the mature VacA toxin takes place. **B-** *vacA* alleles from different *H. pylori* strains show a high level of diversity. Particularly allelic diversity is seen near the 5′ terminus of *vacA* (the s-region) and in the mid-region of the gene (the m-region). It has been reported that there are two main families of s-region sequences (s1 and s2) and two main families of m-region sequences (m1 and m2). *vacA* allele subtypes of s1 include s1a, s1b, s1c, and subtypes m1 and m2 include m1a, m1b, m2a and m2b and certain subtypes have a geographically restricted distribution. Homologous recombination among *vacA* alleles from different strains results in mosaic forms of *vacA* (Adopted from Cover and Blanke, 2005).

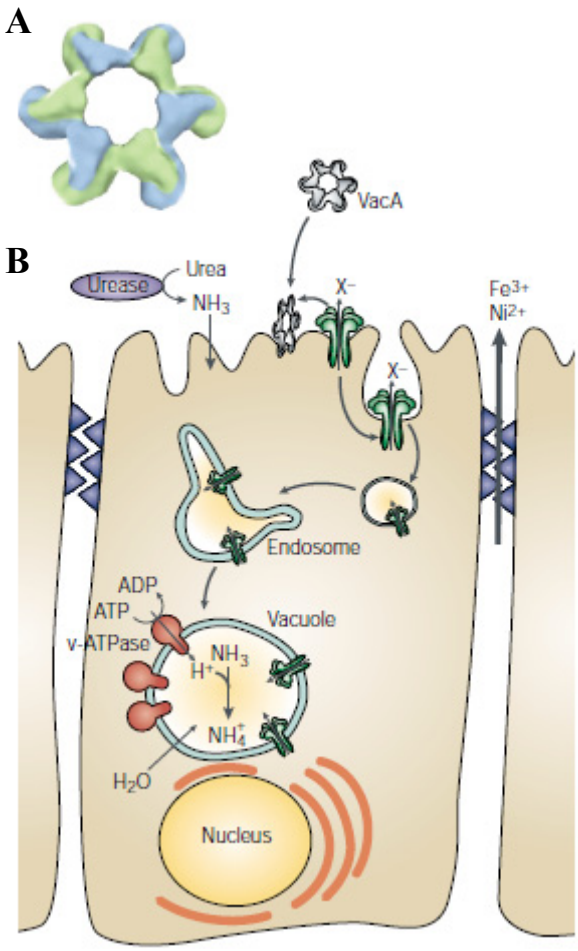

Fig. 1.5AB Cellular vacuolation induced by VacA. **A**-shows oligomeric nature of toxin, and **B-** schematic illustration of a model for the mechanism of VacA-induced vacuole formation. A hexameric anionselective channel of low conductance is formed by the toxin that binds to the apical portion of epithelial cells and inserts into the plasma membrane. Bicarbonate and organic anions released through these channels support bacterial growth. The toxin channels are gradually endocytosed and finally reach late endosomal compartments, increasing their permeability to anions with enhancement of the electrogenic vacuolar ATPase (v- ATPase) proton pump. The ammonia generated by the *H. pylori* urease, in the presence of weak bases, osmotically active acidotropic ions will accumulate in the endosomes. This eventually leads to water influx and vesicle swelling, an essential step in vacuole formation. (Adopted from Cover and Blanke, 2005).

Vaculating activity is high in s1/m1 genotype than in s1/m2 genotype and there is no activity in s2/m2 genotype. Due to high activity in s1/m1 genotype it is more frequently associated with peptic ulceration and gastric cancer. Recently, a new vacA polymorphic site, the intermediate (i) region, has been reported where vacuolation assays showed that i-type determined vacuolating activity among s1/m2 strains (Rhead *et al*., 2007). In the mature toxin, the p33 domain near the N-terminus of VacA contains only

one strongly hydrophobic region (McClain *et al*., 2003). Three tandem GXXXG motifs (defined by glycine residues at positions 14, 18, 22 and 26) (Cover and Blanke, 2005; McClain *et al*., 2003) are contained in this region that is characteristic of transmembrane dimerization sequences.

VacA has a role in membrane channel formation in epithelial cell and is required for VacA-induced cell vacuolation thus inducing the release of urea and anion from the host cells (Fig 1.5B). Transcellular permiablity is also increased which leads to the release of nutrients and cation. Interestingly, a significant part of toxin remains associated with the outer membrane of *H. pylori* and is not released into the environment (Fitchen *et al*., 2005). These toxin clusters are transferred to the host cell surface and exert their toxic action when bacteria contact with host cells. The involvement of specific receptors that mediate the bacterium-cell contact (Fitchen *et al*., 2005) has been suggested by this contact–dependent direct delivery mechanism.

6. Diversity of genome

In comparison to most other bacterial species *H. pylori* genome displays an unusual degree of diversity. The variability between strains was initially indicated by the restriction endonuclease digest analysis of genomic DNA. Using the same technique other researchers have subsequently substantiated a high level of diversity in a number of isolates world wide (Owen, 1995). A high degree of diversity between strains of the *H. pylori* has also been confirmed by analyses of the genome by a variety of other molecular techniques. Prominent among these are profiling of rRNA gene (Owen, 1995), fingerprinting based on polymerase chain reaction (PCR) (Akopyanz *et al*., 1992) and macro restriction digest profiling by pulsed field gel electrophoresis (Taylor *et al*., 1992). These data have clearly indicated the apparent colonization by their own particular genomic variant of *H. pylori* in most individuals. It has been demonstrated that strains undergo genome rearrangements after they infect a new human host however, the reasons for the genetic diversity observed among *H. pylori* strains remain to be explained. The stresses associated with colonization and adaptation to a new environment may be responsible for this (Taylor *et al*., 1992). A number of factors may be involved in this variation such as the movement of short repetitive DNA sequences, uptake of DNA by natural transformation or silent mutations, which is combined with a high rate of recombination events. The degree of change observed within almost every part of the

genome so far analysed cannot be attributed to a single factor and yet the genome size, overall inter-strain DNA sequence homologies, base composition and phenotype appear to be conserved. A high rate of genetic variation due to mutation such as 'silent' point mutation may be the mechanism that enables persistent infection of *H. pylori* for many years in a hostile environment. Till date no studies have been made for monitoring the evolution of a *H. pylori* strain over a period of several years *in situ* in the gastric mucosa. However, evidence suggests that the same genotype was maintained in one strain in an individual who had been subjected to endoscopy on four separate occasions over a period of 9 month. This occurred between and after two different treatment regimens. Laboratory maintained strains show evidence on the stability of the genome even on being subjected to the stress of lyophilization and frequent subculture under laboratory conditions.

7 Contribution of host genetics

It has been reported and now felt that not only the characteristics of the pathogen factor and environmental factor but also host genetics play an important role in determining susceptibility and severity of *H. pylori* infections (Fig. 1.6). It is known that an array of pro- and anti-inflammatory cytokines regulate this inflammation. Genetic polymorphisms directly influence inter individual variation in the magnitude of cytokine response, the expression levels of gene products by generation or deletion of transcription factor sites or by affecting RNA splicing and subsequent translation. It is speculated that the most relevant candidate genes would be ones whose products were involved in handling the *H pylori* infection and resulting inflammation. These candidate genes would be prohibitively extensive, the initial search focuses on genes that are most relevant to physiology of gastric and, in particular, gastric acid secretion. Three main gastric phenotypes have been observed, and each is associated with a set of pathophysiologic abnormalities. The most common phenotype by far, is known as "simple or benign gastritis" phenotype and characterized by mild pangastritis with little disruption of gastric acid secretion (Amieva and El-Omar, 2008). This phenotype is observed in subjects who have no symptoms (asymptomatic) and they develop no serious gastrointestinal (GI) disease. The second phenotype is duodenal ulcer and this phenotype is characterized by an antral-predominant pattern of gastritis with relatively limited acid producing corpus mucosa. This phenotypic subject has high antral inflammation, high gastrin, very high acid secretion and relatively healthy corpus mucosa, all these pathophysiologic changes lead to development of peptic ulcer (El-Omar *et al.*, 1995). The third phenotype is the

"gastric cancer phenotype," and is the most serious phenotype which is characterized by a multifocal gastric atrophy, corpus-predominant pattern of gastritis and achlorhydria or hypochlorhydria (El-Omar *et al.*, 2001). Interesting part of this story is that subjects who develop duodenal ulcers are protected from gastric cancer development, suggesting that the 2 outcomes are mutually exclusive (Hunt, 1996). Thus, it is clear that in the presence of *H pylori*, an endogenous agent upregulates and has a profound proinflammatory effect and is also an acid inhibitor. Interleukin 1B (IL-1ß) fits this profile perfectly because it is not only most important proinflammatory cytokines in the context of *H pylori* infection but also the most powerful acid inhibitor so far known (El-Omar *et al.*, 2001).

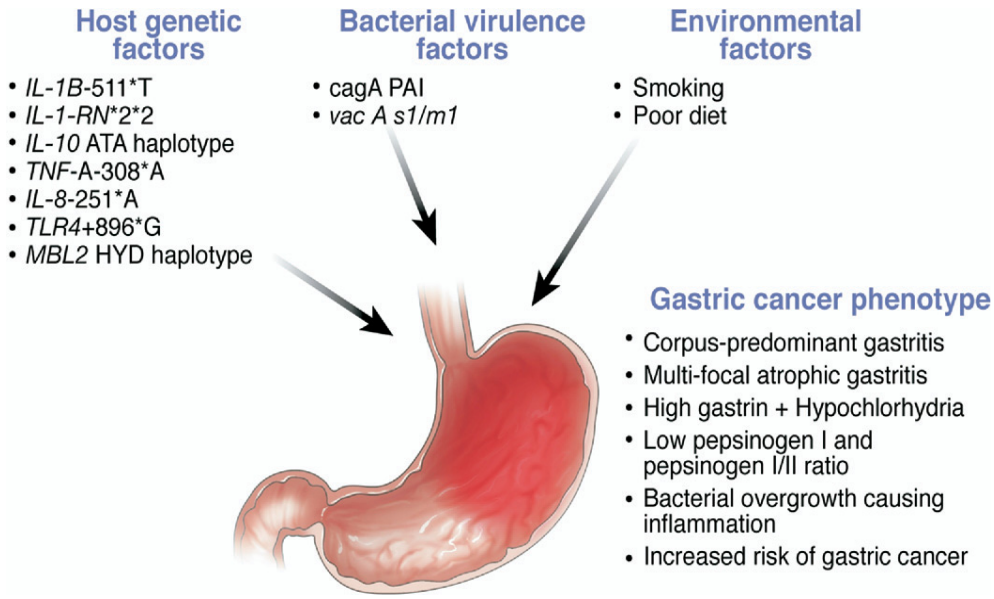

Fig. 1.6 Contribution of host genetic, bacterial and environmental factors to pathogenesis of *H pylori*-induced gastric cancer. An intragastric milieu is created by the combination of these factors that is characterized by chronic inflammation, hypochlorhydria, bacterial overgrowth and sustained genotoxic stress. This might ultimately lead to gastric cancer (From Amieva and El-Omar, 2008).

7.1 Role of IL-1 gene cluster polymorphisms in *H pylori*-induced gastric damage

The expression levels of gene products directly affect genetic polymorphisms by generation or deletion of transcription factor sites or by affecting RNA splicing and subsequent translation. The metabolism of certain compounds is directly influenced or the expression of immune mediators downstream of the gene is affected indirectly with specific polymorphism. Previous studies laid stress on the role of certain mutations in the gene that encodes cytochrome P450, which affects the metabolism of PPIs and antibiotics thus affecting the efficacy of eradication treatment of *H. pylori.* Secondly the effects of mutations in genes encoding antioxidative proteins such as glutathione *S*-transferase (GST) have also been studied (Chen *et al*., 2004; Rollinson *et al*., 2003; Setiawan *et al*., 2001; You *et al*., 2005). However, colonization of *H. pylori* is not directly affected by these mutations. The complex interplay of proinflammatory and anti-inflammatory mediators control and maintain the chronic active inflammation which is responsible for the pathogenic effects of *H. pylori* infection (Macarthur *et al*., 2004). Independent studies conducted in recent years have shown the role of *H. pylori* in gastric disorders and the genetic polymorphisms that affect the expression levels of these inflammatory mediators. Thus it can be concluded that IL-1 gene is related to increased risk of developing gastric cancer which is caused by proinflammatory genetic polymorphisms. A gene cluster which contains the polymorphic IL-1B (encoding the IL-1β cytokine) and IL-RN (encoding the IL-1 receptor antagonist) genes encode the IL-1 cytokine. The most powerful known inhibitor of acid secretion is IL-1 β which is also a powerful proinflammatory cytokine (Calam, 1999; Falush *et al*., 2001). High-level expression of IL-1 β is enabled by the IL-1 gene cluster that contains several polymorphisms, such as IL-1B-31C/C, IL-1B-511T/T, and IL-1RN 2/2. This eventually contributes to decline in acid production, which is related to corpus-predominant colonization by *H. pylori*, resulting the formation of atrophic gastritis, pangastritis, and increased risk of gastric cancer (El-Omar *et al*., 2000, 2001, 2003). Polymorphisms in other inflammation-associated genes e.g., the genes encoding tumour necrosis factor alpha (TNF-α) and IL-10 show similar effects. TNF-β is a proinflammatory cytokine, and TNF-A gene shows several polymorphisms.

7.2 Other cytokines

Increased TNF-α production, is associated with the TNF-A* 308A genotype which along with IL-1, influences the production of gastrin and consequentially affects acid production by gastric parietal cells (Suzuki *et al*., 2001). Thus the association of TNF-A*308A genotype with *H. pylori* gives rise to infection and increases the risk of gastric cancer (El-Omar *et al*., 2003; Ohyama *et al*., 2004; Rad *et al*., 2004; Zambon *et al*., 2005). Likewise, the IL-10 gene haplotypes affect the expression of the anti-inflammatory cytokine IL-10. Higher expression level of IL-10 is related to the GCC haplotype and hence gives rise to anti-inflammatory response, whereas decreased level of IL-10 is related to the ATA haplotype which favours a shift toward a proinflammatory response (El-Omar *et al*., 2003; Hamajima *et al*., 2003; Hellmig *et al*., 2005; Rad *et al*., 2004; Wu *et al*., 2003; Zambon *et al*., 2005). The GCC haplotype favours colonization with more-virulent *H. pylori* strains (Rad *et al*., 2004), whereas increased risk of gastric cancer is related to the ATA haplotype (El-Omar *et al*., 2003; Hamajima *et al*., 2003; Hellmig *et al*., 2005; Lu, *et al*., 2005; Zambon *et al*., 2005). Several independent studies have established that vulnerability to development of gastric cancer is increased only two- to three fold by single polymorphisms while the presence of multiple proinflammatory genotypes increases it substantially (El-Omar *et al*., 2003; Lu, W., *et al*., 2005; Zambon *et al*., 2005). Overall, the increased risk of development of gastric cancer cannot be attributed to a specific polymorphism, as much depends on the vulnerability to *H. pylori* (Queiroz, *et al*., 2004; Rad *et al*., 2003; Zambon *et al*., 2002). Eventually the disease outcome is jointly contributed to factors like the interaction between the different pro- and anti-inflammatory polymorphisms, the immune status of the host, and the characteristics of the colonizing *H. pylori* strain.

The lack of information about the diversity of *H. pylori* specifically isolated from North India stimulated our interest to carry out research so as to have a better understanding of the proposed objectives. The work was undertaken with the following objectives;

Objectives

ENVIRONMENTAL FACTORS

1. **To evaluate the role of environmental factors in causing gastritis, ulcer and gastric cancer in relation to food habits and mode of living.**

PATHOGEN FACTORS

2. **To develop a simple and rapid diagnostic method for the characterization of the most important pathogenicity gene employing culture independent approach since *H. pylori* is a fastidious organism.**

3. **To study genetic diversity among different isolates (from Varanasi and Hyderabad) using ARDRA, ERIC-PCR, AFLP and sequencing of 16S rDNA.**

4. **Genotyping and diversity analysis of *H. pylori* based on known pathogenic genes namely, *cagA*, *cagE* and *cagT*.**

HOST FACTORS

5. **To study the role (s) of host factors on the risk of gastric cancer due to the polymorphisms of IL-1β and IL-1RN during infection of *H. pylori.***

ENVIRONMENTAL FACTORS

Survey of patients having different types of gastrointestinal diseases in relation to their food habits and mode of living

Chapter 2

ROLE OF ENVIRONMENTAL FACTORS

Gastrointestinal Diseases: Relation to Food Habit and Mode of Living

1 Introduction

Helicobacter pylori is a pathogen that is believed to be a major risk factor in the development of duodenal ulcer and gastritis. Chronic gastritis caused by *H. pylori* infection, perhaps alongwith exposure to dietary and environmental mutagens, ultimately leads to gastric cancer. Most infections are apparently acquired in childhood and several factors have been postulated for this early acquisition of the infection, including genetic predisposition (Kunstmann *et al*., 2002), immature immune system, disturbed oesophageal gastroduodenal motility, closer person to person contact and overcrowding, lower standards of personal hygiene (Parsonnet, 1995), and poor nutritional state (Goodman *et al*., 1997). Overcrowded housing (Hammermeister *et al*., 1992) and contact with infected spouses and children (Singh *et al*., 1999) are few of the recognized general population adult risk factors for infection. There is inconsistent evidence that males (Lin *et al*., 1998), those who have poor dental hygiene, smoke or consume excessive alcohol (Giannuzzi *et al*., 2001), are prone to infection. Though human host biological abnormality needs not be present for the successful colonization of the gastric mucosa by *H. pylori*, the risk of successful infection is increased by alteration to the integrity of the normal structure and function of the upper gastrointestinal tract. *H. pylori* is transmitted from person to person predominantly via the oral–oral route (Mitchell, 1999).

H. pylori has been classified as class 1 carcinogenic agent by the World Health Organization (IARC, 1994) and it is generally accepted that chronic *H. pylori* infection may lead to peptic ulcer disease and gastric cancer. Based on available data it has been suggested that 6 to 20% of people with *H. pylori* infection develop peptic ulcer disease over a lifetime, which is at least three to four times higher than in noninfected subjects (Tytgat and Dixon, 1993), and 1–3% of persons infected with *H. pylori* develop gastric cancer. In patients with severe gastric atrophy, corpus predominant gastritis, and intestinal metaplasia, changes which occur with a longer duration of infection are predisposed to the risk of gastric cancer (Kuipers *et al*., 2006). It has been reported that patients with *H.*

pylori-caused duodenal ulcers are not predisposed to gastric cancer where as patients with *H. pylori*-positive nonulcer dyspepsia (gastritis), gastric ulcers, and gastric hyperplastic polyps are predisposed to gastric cancer development (Uemura *et al*., 2001). *H. pylori* strain types, long duration of infection, and poor-quality high-salt nutrition are the other cofactors in the development of *H. pylori*-caused gastric cancer.

2 Materials and methods

2.1 Subjects / patients

This study was conducted at the Department of Gastroenterology, S. S. Hospital, Banaras Hindu University, Varanasi, India, This hospital/ medical college is situated in North India and provides territory level health care. The study involved 276 North Indian patients who visited hospital during the period March 2007 to February 2008. Patients with gallbladder, liver, pancreatic, renal and peritoneal disease as well as parasitic infestation were excluded from this study. Only gastritis, ulcer (including gastric ulcer and duodenal ulcer), GERD and gastric cancer patients were selected.

2.2 Endoscopy and case selection

Detailed history of all the patients was taken. Upper GI endoscopy was done after overnight fast. The endoscope (Olympus, CV-70 Videoscope, Japan) and biopsy forceps were well washed and disinfected. Unless otherwise stated endoscope forceps and fibre optics were sterilized in 2 % glutarldehyde solution for 20 min followed by thorough washing in sterilized distilled water. Endoscopic findings were classified according to criteria described by Armstrong *et al*. (1991) (oesophagitis)," Johnson *et al*. (2000) (hiatus hernia), Myren and Serck-Hanssen (1974) (endoscopic gastritis and gastroduodenal reflux), Nesland and Berstad (1985) (erosive prepyloric changes), Venables (1988) (duodenitis), and Bernersen *et al*. (1990) (peptic ulcer and deformed duodenal bulb). Biopsy specimens for analysis were taken from the proximal and distal parts of the duodenum, from both the greater and lesser curvatures of the corpus and antrum of the stomach, and from all lesions as well. Biopsy specimens for culture were also taken from the lesser and greater curvature of the antrum about 3 cm proximal to the pyloric ring.

2.3 Data collection

A questionnaire was created to serve the purpose of the survey (Chart 2.1). To enforce good response rate, we emphasized that the survey was not a test of knowledge but an assessment of opinions. The questionnaire was anonymous and its completion took about 15 minutes.

Chart 2.1 Questionnaire meant for survey of gastrointestinal diseases with relation to food habit and mode of living of patients

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Serial No……. **Sample ID……..** **Date……**

☐Hospital ☐Rural Area ☐Urban Area

Patient information

Patient's Name …….

Age_____ (Month/Years) Sex _____ (M/F)

Occupation _____

Address_____

_____

Phone No._____ Religion_____

Complication_____

Parent's information

Father's Name_____ Occupation _____

Mother's Name_____ Occupation _____

Habitual information

1. From how long you suffered from gastric problem? _____

2. Had your gestroendoscopy been performed previously?

☐No ☐Yes _____

3. No of members in your family …… No of room……

Ratio of members per Room…….

4. Do you take antioxidant food (lemon, gooseberry, orange etc) regularly or occasionally?

☐No ☐Occasionally ☐Yes _____

5. Schedule of taking food

☐Timely ☐Untimely

6. Source of drinking water_____

☐Well ☐Hand pump ☐Supply water ☐Other_____

7. Do you smoke cigarette / Bidi / others alike?

☐No ☐Occasionally ☐Yes_____

8. You wash your teeth with _____

☐Gul / Gurakhu ☐Ash ☐Herbal Stick ☐Tooth paste

9. Do you exercise?

☐No ☐Occasionally ☐Yes_____

Signature of patients _____ **Date**_____

3 Results

3.1 Microbiological analysis

The study was based on 276 patients who went for upper gastroendoscopic examination. The mean age was 47 years, the youngest was 15 years old and the oldest was 80 years. The maximum number of patients was in the age group of 31 to 40 years and the least number of patients was in the age group of 11 to 20 years. Among the 276 patients, 189 (68.5%) was male and 87 (31.5%) was female (Table 2.1). *H. pylori* was present in 226 (81.9%) patients wherein the maximum number of patients was in the age group of 31 to 40 years (88.2%). The lowest prevalence of *H. pylori* was found in the age group of 11 to 20 years (57.1%) which increased with age group of 31 to 40 years and then decreased till the age group of 71 to 80 years (Table 2.2). On the basis of endoscopic findings 60 patients were diagnosed with gastritis, 92 had DU, 62 had cancer and 62 cases were normal. Highest prevalence of *H. pylori* was observed in DU followed by gastritis and cancer and the least in normal (Table 2.3).

Impacts of certain environmental factors on the occurrence of disease were also studied. The prevalence, odds ratio and 95% CI for various factors are represented in Table 2.4.

Table 2.1 Age group and gender of the patients who underwent endoscopy

Age Group (Years)	Gender		Total
	Male	Female	
11 to 20	7	0	7 (2.5)
21 to 30	36	18	54 (19.6)
31 to 40	44	24	68 (24.6)
41 to 50	40	23	63 (22.8)
51 to 60	27	14	41 (14.9)
61 to 70	21	7	28 (10.1)
71 to 80	14	1	15 (5.4)
Total	**189**	**87**	**276**

Value in parenthesis denotes %

Table 2.2 Relationship between age group and prevalence of *H. pylori*

Age Group	No of cases	Prevalence of *H. pylori*
11 to 20	7	4 (57.1)
21 to 30	54	45 (83.3)
31 to 40	68	60 (88.2)
41 to 50	63	52 (82.5)
51 to 60	41	33 (80.5)
61 to 70	28	22 (78.6)
71 to 80	15	11 (73.3)
Total	**276**	**226**

Value in parenthesis denotes %

Table 2.3 Prevalence of *H. pylori* in various types of gastrointestinal complications

Disease	No of cases	Prevalence of *H. pylori*
Normal	62	34 (54.8)
Gastritis	60	54 (90.0)
DU	92	86 (93.5)
Cancer	62	52 (83.9)
Total	**276**	**226(81.9)**

Value in parenthesis denotes %

3.2 Duration of disease symptoms

History of patients revealed that 137 had gastric problems for less than one year and 21 patients had problems since 6 to 10 years. The prevalence of *H. pylori* was higher (86.2%) in patients who had symptoms for more than 11 years with an odd ratio of 1.752 (95% CI 0.566-5.427), p= 0.326 compared to the control (78.1%) patients who had infection for less than one year (Table 2.4).

Table 2.4 Impact of various factors on the prevalence of *H. pylori*-mediated infection

Factors	No of subjects	*H. pylori* positive subjects (%)	Odds ratio (CI 95%)	p value
Duration of gastric problem				
<1 Year	137	107 (78.1)	-	
1 - 5 Years	68	58 (85.3)	1.626 (0.743-3.561)	0.221
6 - 10 Years	21	18 (85.7)	1.682 (0.464-6.096)	0.424
≥ 11 Years	29	25 (86.2)	1.752 (0.566-5.427)	0.326
Unknown	21	18 (85.7)	1.682 (0.464-6.096)	0.424
Gastroendoscopy performed earlier				
No	209	168 (80.4)	-	
Yes	61	54 (88.5)	1.883 (0.798-4.441)	0.143
Unknown	6	4 (66.7)	0.488 (0.086-2.757)	0.408
Number of family members				
1 to 5	88	65 (73.9)	-	
6 to 10	128	109 (85.1)	2.030 (1.028-4.010)	0.039
≥ 11	60	52 (86.7)	2.300 (0.951-5.563)	0.060
Smoking habits[a]				
No	182	153 (84.0)	-	
Yes	83	64 (77.1)	0.638 (0.334-1.220)	0.173
Occasionally	11	9 (81.8)	0.853(0.175-4.152)	0.844
Intake of citrus fruits (juices)[b]				
No	178	161(90.4)	-	
Yes	56	35(62.5)	0.176(0.084-0.368)	<0.001
Occasionally	30	24(80.0)	0.422(0.152-1.177)	0.091
Unknown	12	6(50.0)	0.106(0.031-0364)	<0.001
Sources of drinking water[c]				
Hand Pump	153	122(79.7)	-	
Supply Water	48	42(87.5)	1.925(0.7532-40919)	0.166
Well	29	26(89.6)	2.383(0.679-8.366)	0.164
Others	26	20(76.9)	0.917(0.341-2.468)	0.863
Unknown	20	18(90.0)	2.475(0.546-11.213)	0.226

Table 2.4 Continued

Factors	Number of subjects	*H. pylori* positive subjects (%)	Odds ratio (CI 95%)	p value
Schedule of taking food[d]				
Timely	134	110(82.1)	-	
Untimely	137	112(81.7)	0.977(0.252-1.815)	0.942
Unknown	5	4(80.0)	0.873(0.093-8.160)	0.905
Dental habit[e]				
Herbal stick[1]	92	72(78.2)	-	
Toothpaste[2]	105	92(87.6)	1.966(0.916-4.217)	0.079
Both (1and 2)	23	20(77.8)	1.852(0.499-6.868)	0.351
Gul, Gurakhu	15	13(86.9)	1.806(0.376-8.670)	0.455
Others	8	7(87.5)	1.944(0.226-16.744)	0.538
Unknown	33	22(66.7)	0.565(0.231-1.335)	0.186
Exercise habits[f]				
No	108	88(81.5)	-	
Yes	49	40(81.6)	1.010(0.423-0.413)	0.982
Occasionally	60	49(81.7)	1.012(0.448-2.286)	0.976
Unknown	59	49(83.0)	1.114(0.483-2.568)	0.801

[a] Smoking habits: **Yes**-smoke bidi/ cigarete at least once daily; **occasionally**-smoke 2-3 times in a week, and **No**-never smoked
[b]Citrus fruits (juices): **Yes**- taking at least once daily; **occasionally**-taking 2-3 times in a week
[c]Sources of drinking water: **Other**-water purified and disinfected by reverse osmosis, UV radiations, boiled water etc
[d]Schedule of taking food: **timely**- intake of food timely every day; **untimely-** irregular habit of taking food
[e]Herbal stick: **Both**-Use of herbal stick and/or toothpaste; **Other**-wash of teeth with ash/salt etc
[f] Exercise habits:**Yes**- do exercise or do physical work regularly; **occasionally- No** regular habit of exercise, and No- never do excercise

3.3 Gastroendoscopy performed earlier

Results showed that *H. pylori* was present in 54 out of 61 patients who under went endoscopy earlier where as the number was 168 (out of 209) who did not undergo endoscopy earlier. Analysis of results further revealed that the prevalence of *H. pylori* was higher in patients who underwent endoscopy earlier (88.5%) with an OR of 1.883

(95%CI=0.798-4.441), p=0.143 compared to those patients where endoscopy was not performed earlier (80.4%) (Table 2.4). The maximum number of patients who had their endoscopy performed earlier was diagnosed with DU (28.3%). On the other hand the number of patients who never had their endoscopy performed earlier was highest and had no symptoms of disease (87.1%) (Fig. 2.1).

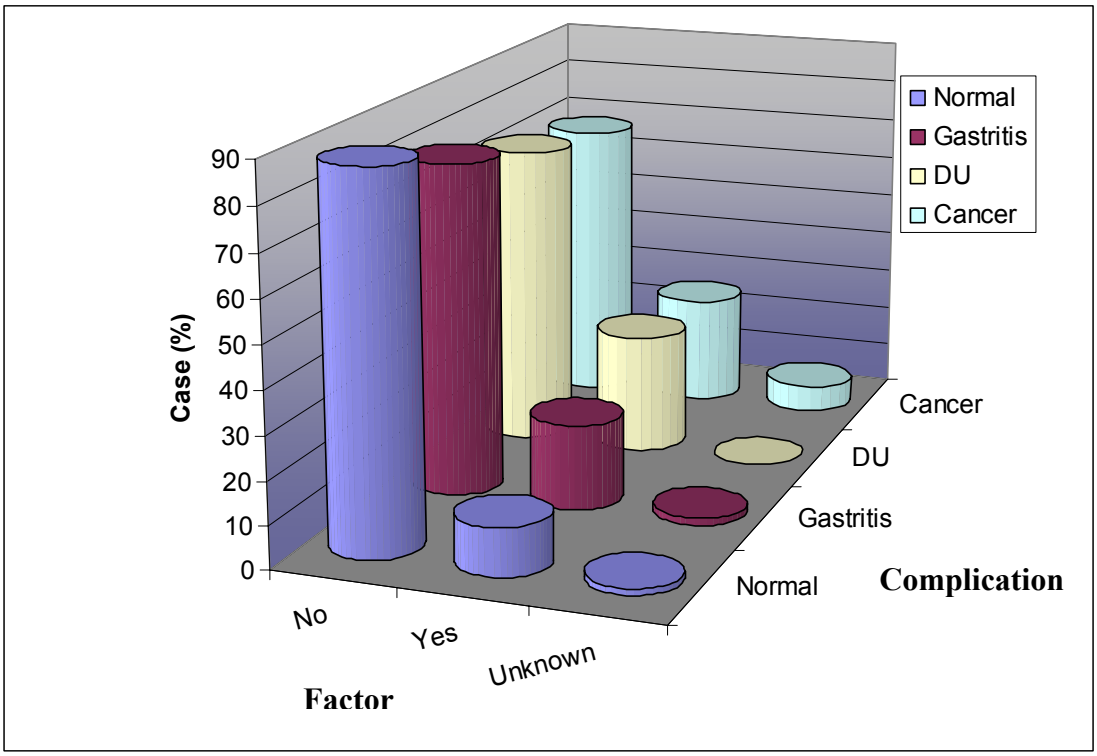


Fig. 2.1 Distribution pattern of various types of gastrointestinal complications in patients who underwent gastroendoscopy earlier.

3.4 Number of family members

Survey conducted showed that the maximum number of patients (128) had 6 to 10 members in their family, 60 patients had more than 11 members in their family. The prevalence of *H. pylori* was detected highest (86.7%) in a family having more than 11 members with an OR of 2.300 (95%CI=0.951-5.563), p=0.060; compared to 73.9% in control group with 1 to 5 members (Table 2.4).

3.5 Smoking habits

An attempt was made to find out the effects of smoking on the appearance of various types of gastric diseases. Results showed that 83 patients smoked regularly, out of which 182 was non- smoker and 11 patients smoked occasionally. The prevalence of *H. pylori* was highest (84.0%) among non -smokers and the lowest in smokers (77.1%). However compared to non smokers, smokers had an OR of 0.638 (95%CI= 0.334-1.220), p=0.173 (Table 2.4).

3.6 Intake of citrus fruits (juices)

Impact of citrus fruits intake like lemon, oranges etc by the patients on the gastrointestinal diseases was also studied. Survey conducted showed that 56 respondents had habit of citrus fruits intake regularly while 178 never had it (Table 2.4). Highest prevalence of *H. pylori* was noted in patients (90.4%) who never had fruit juice intake, the value was least in the patients (62.5%) who had regular intake of juice with an OR of 0.176 (95%CI=0.084-0.368), p<0.001. Clinically maximum number of patients (27.2%) having regular intake of citrus fruits belonged to DU group and the lowest number was noted in cancer (6.5%) group. Among the patients who never had citrus fruits intake, the case of gastritis was minimum (48.3%) and the maximum patients were normal (75.81%) (Fig. 2.2).

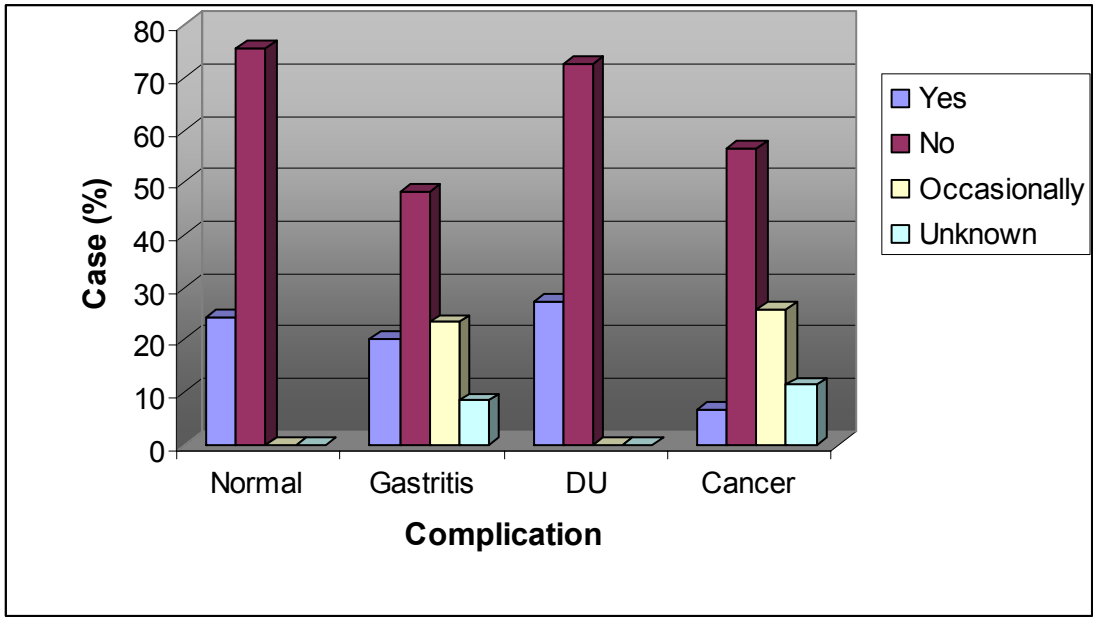


Fig. 2.2 Relationship between complication types and intake of citrus fruit juice (anti-oxidant supplement).

Yes-Intake at least once daily
No-No intake for months
Occasionally-Intake at least once weekly/monthly
Unknown-Data not available

3.7 Sources of drinking water

Role of drinking water source on the disease appearance was surveyed in different patients. Highest prevalence (89.6%) of *H. pylori* was noticed in patients who used well water for drinking purpose with OR of 2.383 (95%CI=0.679-8.366), p=0.164 and the least prevalence was observed in the patients who used other sources of water with OR of 0.917(95% CI=0.341-2.468), p=0.863 (Table 2.4). Interestingly patients depending on hand pump water for drinking purpose were found normal. Notably the maximum number of patients who consumed supply water had cancer (21%) and those patients depending on well water had gastritis (18.3%). In the case of other sources of drinking water, the maximum number of patients (16.1%) was normal (Fig. 2.3).

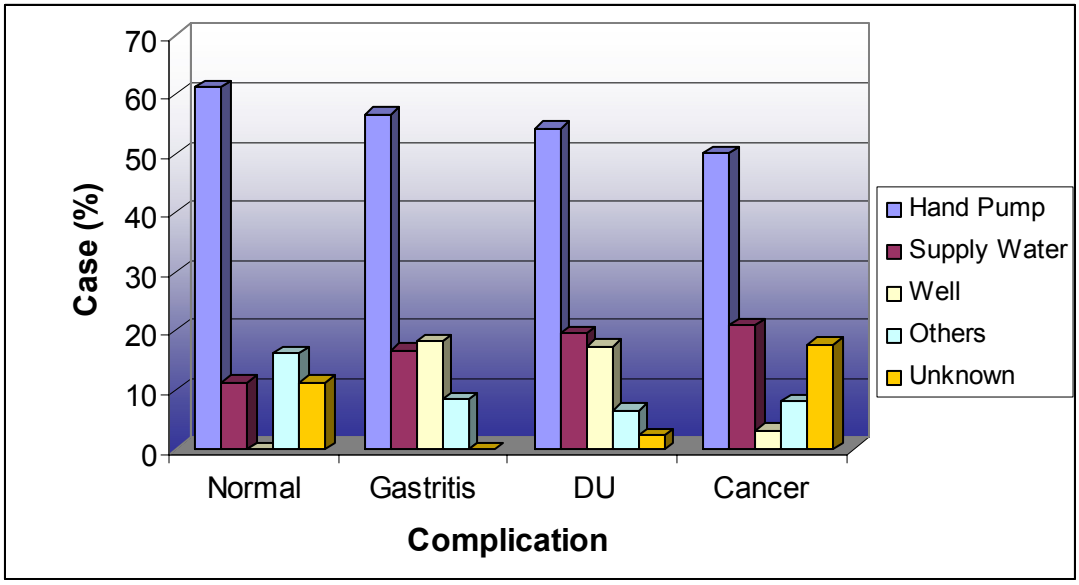


Fig. 2.3 Impact of drinking water source on various types of gastrointestinal diseases.

Other-purified and disinfected by reverse osmosis, UV radiations, boiled water

3.8 Schedule of taking food

Survey conducted for the schedule of food intake habit showed that the number of patients eating timely or untimely was almost same. Similarly the prevalence of *H. pylori* in patients taking food timely or untimely was also more or less similar. The OR of patients eating food untimely compared to those eating timely was 0.977 (95% CI=0.252-

1.815), p=0.942 (Table 2.4). Clinically maximum number of patients eating timely were normal (58.1%) and the least had cancer (43.5%). In the case of patients eating untimely, the maximum number of patients had DU (53.3%) while 41.94% of patients was found normal (Fig. 2.4).

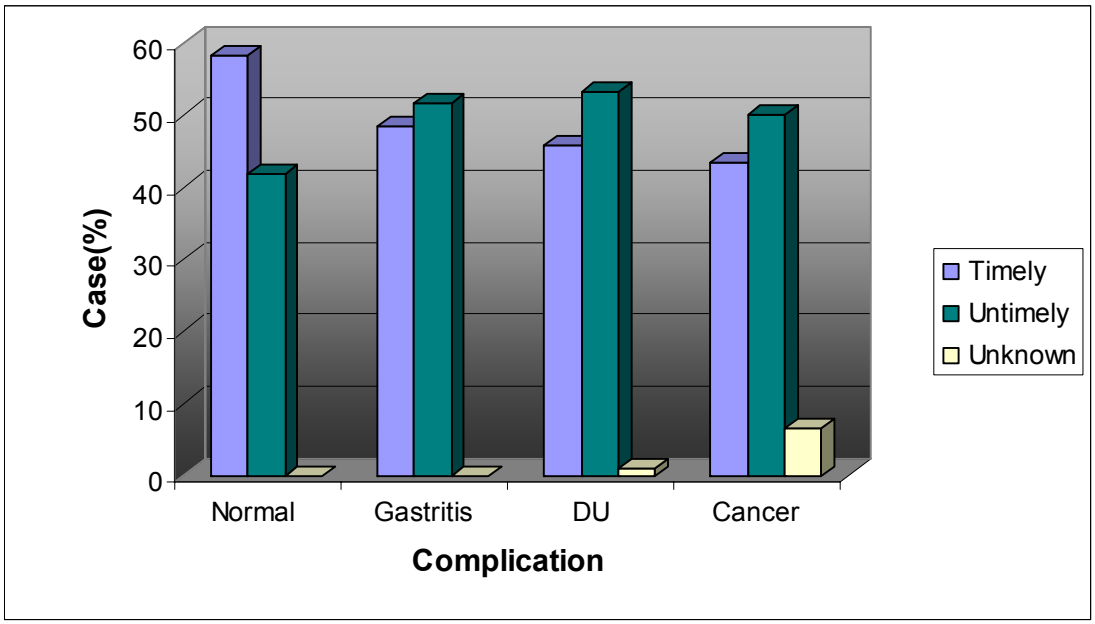


Fig. 2.4 Relationship between complication types and schedule of food intake habit.

3.9 Dental habits

History of patients revealed that maximum number of patients (n= 105) used toothpaste. Highest prevalence of *H. pylori* was noted in patients using toothpaste with an OR of 1.966 (95%CI=0.916-4.217), p=0.079 (Table 2.4). Prevalence of *H. pylori* was least in the patients using both toothpaste and herbal stick with OR of 1.852 (95%CI=0.499-6.868), p=0.351. Clinical symptoms revealed that among the patients using toothpaste maximum had cancer while patients using herbal stick had maximum cases of DU. Maximum number of patients (14.5%) using both toothpaste and herbal stick belonged to normal group while patients using gul/ gurakhu (16.1%) had cancer (Fig. 2.5).

3.10 Exercise habits

Survey of cases revealed that among all the patients 49 had habit of doing exercise regularly, 108 never did exercise and 60 performed exercise irregularly. Prevalence of *H. pylori* was almost equal in all the groups of patients e.g., exercised regularly (81.6%), occasionally (81.7%) and those who did not exercise at all (81.5%) (Table 2.4). Clinically maximum number of cases exercising regularly was normal (25.8%) while patients who did not exercise at all were diagnosed with cancer. Interestingly patients exercising occasionally (40.2%) were diagnosed with DU symptoms (Fig. 2.6).

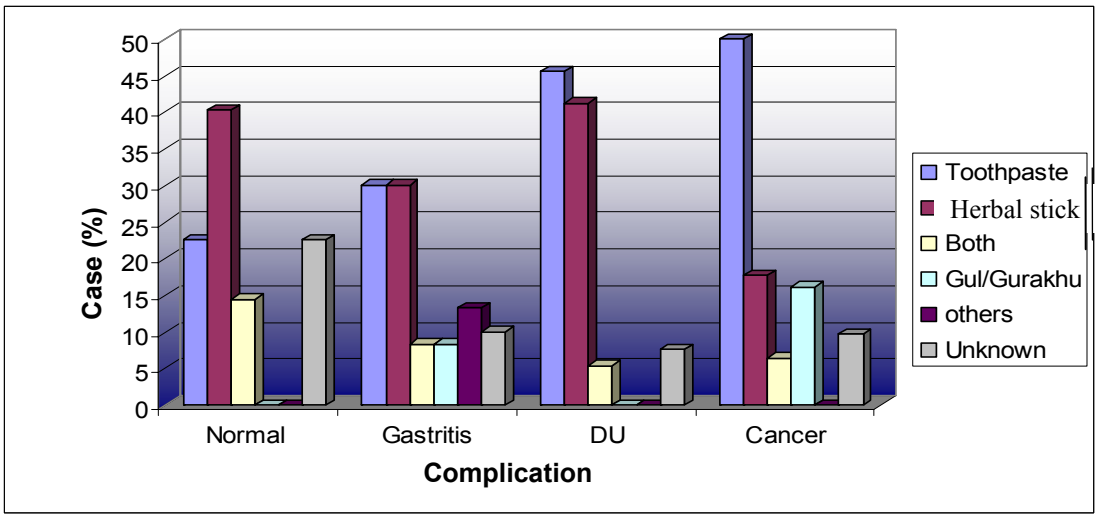


Fig. 2.5 Effects of dental habits on the appearance of gastrointestinal complications.

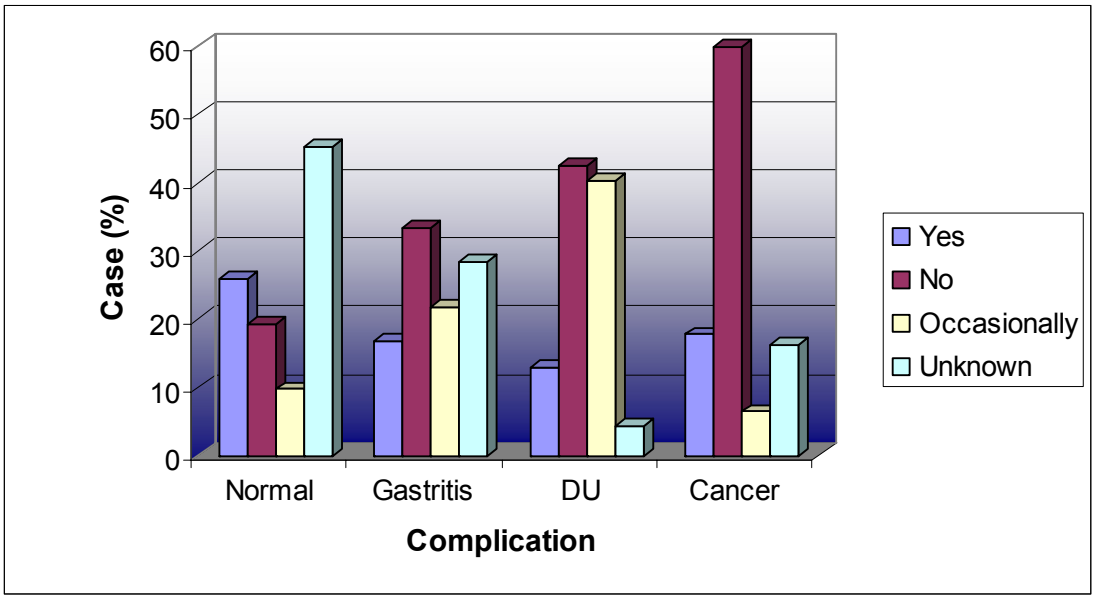


Fig. 2.6 Impact of exercise habits on the distribution pattern of various types of gastroentestinal complications.

4 Discussion

At global level *H. pylori* is a challenging health problem with the prevalence of *H. pylori* infection varying worldwide, with less than 40% prevalence in developed countries, and more than 80–90% in developing countries (Correa, 1996; Zhang *et al*., 1996). In recent years, correlation between *H. pylori* infection and risk factors has been investigated around the world (Chen *et al*., 2005; Goodman *et al*., 2003; Moayyedi *et al*., 2002). However contradictory results have been reported by different workers although there is a general opinion that *H. pylori* infection is related to socioeconomic status (Huang *et al*., 2004; Koch *et al*., 2005; Kurzeja-Miroslaw *et al*., 2004). Till date the risk factors of *H. pylori* infection are still poorly understood (Bures *et al*., 2006; Malaty and Nyren, 2003; Perez-Perez *et al*., 2004). Survey conducted in this study is probably the first in India dealing with the effects of different factors on the prevalence of *H. pylori* and the occurrence of different gastro- intestinal diseases. Results of this study clearly showed the prevalence of *H. pylori* in 81.9% patients of this region. The percentage of female patients was lesser than (31.5%) the male patients (68.5%) (Table 2.1). This might be due to the fact that in general women tend to live protected lives in homes with good sanitation facilities while men usually engage in business that brings them in contact with general population and to less protected sources of food and water (Ramirez-Ramos *et al*., 1990).

Our findings show that the prevalence of *H. pylori* increases with age till 31 to 40 years group and then decreased (Table 2.2). Malcolm *et al*. (2004) have also reported that *H. pylori* infection was associated with age, sex, and socioeconomic conditions. There is strong evidence that children acquire *H. pylori* from their parents (Perez-Perez *et al*., 2004) or colonised siblings (Bures *et al*., 2006). Previous studies reported that the prevalence of *H. pylori* infection increased with age, and the prevalence was lower in subjects younger than 20 years old (Aguemon *et al*., 2005; Rodrigues *et al*., 2005). Furthermore prevalence starts decreasing when the patients start taking medicines.

It is interesting to note that the prevalence was least in patients who had symptoms of less than one year and maximum in patients having symptoms of more than 11 years. Prevalence of *H. pylori* was more in patients who had undergone endoscopy earlier in comparison to those who never had undergone endoscopy earlier. From the above observation it appears that the transmission of *H. pylori* might have occurred during

endoscopy although our data is not statistically significant to support above conclusion. Shi *et al*. (2008) have also reported that there was no association between *H. pylori* infection and examination by endoscopy.

Risk factors of *H. pylori* generally include sharing a bed with others in childhood, more siblings, more family members, and lower education status (Bures *et al*., 2006; Farrell *et al*., 2005; Garg *et al*., 2006). Our findings are in accord with above reports, since prevalence was least in patients with 1 to 5 (73.9%) family members but was significantly higher in patients with 6 to 10 family members with an odds ratio of 2.030, p=0.039 (Table 2.4). Furthermore the percentage increased with number of family members, the highest being in patients having more that 11 members in their family with an odds ratio of 2.300 and significant value on borderline, p=0.060. Similar to our findings, Shi *et al*. (2008) have also reported that the prevalence of *H. pylori* infection was associated with more family members, and that the prevalence tended to be higher if there were children younger than 10 years in the family.

Studies conducted earlier have demonstrated that the prevalence of *H. pylori* infection has a negative correlation with consumption of tobacco (Shi *et al*., 2008)**.** This could possibly be due to increased gastric acid secretion by smoking (Ogihara *et al*., 2000). Alike previous reports (Brown *et al*., 2002; Shinchi *et al*., 1997), we noted that *H. pylori* infection was negatively correlated to smoking and the trend was not statistically significant. More or less similar findings have been reported by earlier worker (Shi *et al*., 2008).

Bae *et al*. (2008) have reported that the risk of gastric cancer is associated with citrus fruit intake. Based on the findings that high vegetables and fruit consumption are inversely related to risk of gastric cancer (Fuchs and Mayer, 1995), several scientists have suggested possible presence of anticarcinogenic substances in vegetables and fruits (Gallus *et al*., 2004; Yuan *et al*., 2004). Ascorbic acid (vitamin C) especially, acts as an antioxidant and can quench reactive oxygen species produced in the gastric environment (Drake *et al*., 1996). It has been demonstrated that the effective inhibition of *H. pylori* infection takes place with high dose of vitamin C (Kim *et al*., 2005). In addition to vitamin C, citrus fruits are the main dietary source of β-cryptoxanthin, another major carotenoid in human plasma (Mangels *et al*., 1993), which is known to show protective effect on lung cancer (Mannisto *et al*., 2004). Our findings do support above reports since

we noticed that patients who consume citrus fruits were less prone to cancer than those who did not. The percentage of patients consuming citrus fruits was 6.5% while the percentage of patients who didn't consume citrus fruits was 56.5%. Cases who consumed fruit juice occasionally, the percentage was 25.8%. Altogether the findings show that the prevalence of *H. pylori* infection was maximum (90.4%) in non consumer of citrus fruits and least in consumers (62.5%) and the data is statistically significant $p<0.001$. Our findings are in agreement with those of Bae *et al*. (2008). Additionally, Gonzalez *et al*. (2006) have also found an inverse association between citrus fruit intake and cancer of the stomach.

Transmission of *H. pylori* takes place through two possible routes which include direct and indirect transmission. Direct transmission takes place through person to- person interaction, while indirect transmission takes place through carriers such as water, air, food, flies and other animals (Allaker *et al*., 2002). Since transmission through contaminated water, has been reported for many diseases, a waterborne route is a plausible mode of *H. pylori* infection (Blaser, 1998; Marshall and Warren, 1984). It has been demonstrated by several workers that *H. pylori* can survive in water for long periods of time and that different sources of drinking water show different degrees of prevalence (Bellack *et al*., 2006; Rolle-Kampczyk *et al*., 2004). Presence of *H. pylori* has been reported in surface water and groundwater samples in various parts of the world especially in places where human or animal faecal contamination were more likely (Hegarty *et al*., 1999; Hulten *et al*., 1998; Karita *et al*., 2003; Mazari-Hiriart *et al*., 2001; Sasaki *et al*., 1999). DNA of *H. pylori* has been detected in water pots and in a cast-iron water pipe from a municipal water system suggesting that biofilms may play a role in transmission in real-world settings (Bunn *et al*., 2002; Park *et al*., 2001). Waterborne transmission depends on the viability of *H. pylori* once it enters water and its capability to colonize the human stomach upon ingestion. In developing countries water source has been implicated as a risk factor for *H. pylori* infection. Water from different sources (i.e. municipal vs. private) is variable in quality because of the diversity in environmental influences and water distribution systems. Surveys made by other workers indicate that the prevalence of infection is higher among people drinking untreated water or water from shallow wells (Brown *et al*., 2002), and among people lacking tap water or an internal water supply in the home (Constanza *et al*., 2004; Klein *et al*., 1991; Nurgalieva *et al*., 2002; Olmos *et al*., 2000; Ueda *et al*., 2003).

Results of the present study show that the prevalence of *H. pylori* infection was highest in subjects depending on well water with an odds ratio of 2.383 and least in those having other sources of water with an OR of 0.917. Other sources of water included water purified and disinfected by reverse osmosis, UV radiations, boiled water etc. However the data obtained is not statistically significant. Clinical observations showed that patients dependent on drinking water from hand pump (61.3%) were normal whereas patients using supply water had the highest number (21%) of cancer. Similarly among all the cases using well water for drinking purpose majority had gastritis however maximum cases drinking water from other sources (16.1%) were normal. Our survey results also show that the schedule of taking food was found to have very little effect on the prevalence of *H. pylori*.

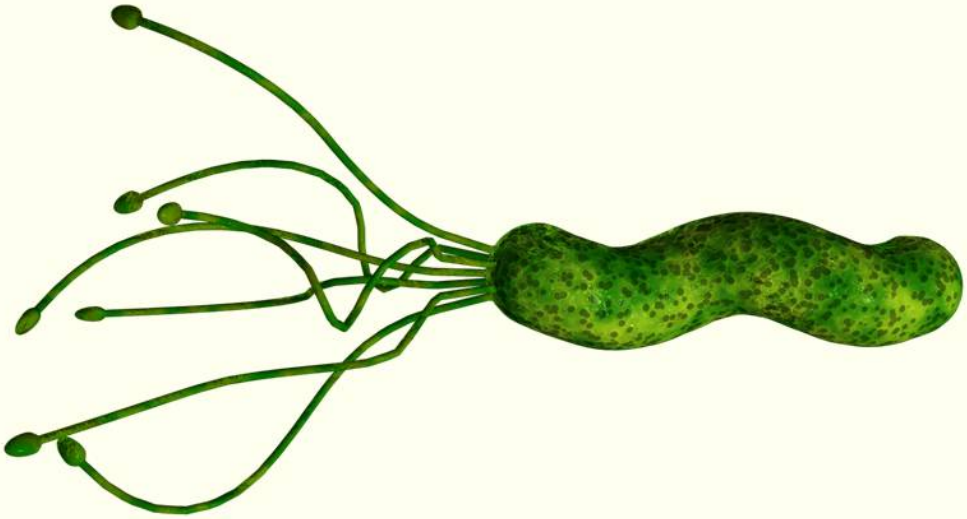
It is interesting to note that the dental habits have significant impact on gastrointestinal complications and the prevalence of *H. pylori*. This is evident from the fact that the prevalence of *H. pylori* was maximum in patients (87.6%) using toothpaste with an odds ratio of 1.966 which points towards low standard of dental care. It is also possible that sharing of toothbrush in some resource poor families and contaminated toothbrush could be the cause of high prevalence. Detailed investigations are needed to arrive at a firm conclusion since the data is not significant statistically.

Yiling *et al*. (2000) reported that physical activity could possibly affect peptic ulcer disease through several biological mechanisms including: (*a*) reducing excess acid secretion; (*b*) enhancing the immune system's ability to neutralize the effects of *H pylori*, and (*c*) improving a person's ability to cope with stressful situations. It has also been reported that regular moderate physical activity is associated with a lower risk of bacterial infections (upper respiratory tract infections) (Nieman, 1998) although experimental studies support an alteration in immune status in both athletes and non-athletes in response to exercise (Woods *et al*., 1999), the issue is debatable and yet to be resolved.

Few studies have reported a decrease in basal or meal stimulated acid secretion with cycling and after exertion, leading to suggestions that exercise may aid in duodenal ulcer healing and maintenance of remission (Markiewicz *et al*., 1977; Meeroff, 1985; Ramsbottom and Hunt, 1974). Regular physical activity (walking, gardening, or vigorous physical activity) has been found to be connected with a decreased risk for severe gastrointestinal haemorrhage in older subjects suffering from gastroduodenal ulcer or

gastritis. We have observed no association between prevalence of *H. pylori* and exercise habits and the data is also statistically insignificant. However, clinically we have observed that patients who exercised regularly had least risk of developing DU (13%), the maximum number of patients were normal (25.8%). Those who didn't exercise at all had the maximum risk of developing cancer (59.7%) followed by DU (42.4%). Those who exercised occasionally had the maximum risk of developing DU (40.2%). Cheng *et al*. (2000) have also reported that physical activity was inversely related to the incidence of duodenal, but not gastric, ulcers for men only.

PATHOGEN FACTORS



Chapter 3

PATHOGEN FACTORS

Section 1

Pathogenicity Gene Status

Evaluation of a Novel Multiplex PCR Technique for Rapid Diagnosis and Characterization of pathogenicity gene

1 Introduction

Helicobacter pylori is a highly successful bacterial pathogen that persistently colonizes the mucosa of the human stomach (Covacci *et al*., 1999; Cover and Blaser 1999; Ernst and Gold, 2000). There is increasing evidence that distinct variants of *H. pylori* exist and that these may be associated with the pathogenicity of the bacterium (Gatti *et al*., 2006; van Doorn *et al*., 1998a); several virulence-associated genes have been identified (Atherton *et al*., 1995; Covacci *et al*., 1993).

Virtually, the presence of *vacA* gene has been reported in all *H. pylori* strains; various strains show marked differences in production of vacuolating cytotoxins (Mahboob *et al*., 2005). It is assumed that the differences might be due to variations in cytotoxin structure, the regions of highest diversity being localized at the N-terminal part of the toxin (corresponding to 2 different toxin signal sequences s1/s2) and in the mid-region of v*acA* (m1/m2, corresponding to two different mid-regions of the toxin that are required to bind different cell types). The v*acA* genotype s1/m1, unlike s2/m1 (a form of v*acA* associating the signal sequence type 2 with the mid-region type 1), can vacuolate cells. It has been reported that because of the presence of an additional sequence of 12 amino acids at the N-terminal end of s2, v*acA* toxin is inactivated (Atherton *et al*., 1995; Letley *et al*., 2003). The amino acid sequences of type m1 and m2 VacA proteins are ~65% identical within a region of 250 amino acids (Atherton *et al*., 1995). Recently, a new *vacA* polymorphic site, the intermediate (i) region, has been reported where vacuolation assays showed that i-type determined vacuolating activity among s1/m2 strains (Rhead *et al*., 2007).

Another important gene, *cagA* (120- to 145 kDa protein) is a highly antigenic protein that is associated with a prominent inflammatory response by eliciting interleukin-8 production (Atherton, 2000). *cagA* is located at one end of the *cag* pathogenicity island (*cag*PAI), a 40-kb DNA fragment that also encodes molecules constituting the bacterial type IV injection system (Yokoyama *et al*., 2005). Several workers have followed *cagA* genotyping for distinguishing various strains of *H. pylori* (Kaklikkaya *et al*., 2006; Matteo *et al*., 2007; Rejane *et al*., 2007).

Because the bacterium is fastidious and microaerophilic, researchers especially from the resource-poor developing countries face problems in *in vitro* culture and growth. Besides, methods developed so far for detecting *H. pylori* infection, including the *H. pylori* stool antigen test (Cardinali *et al*., 2003) and PCR restriction analysis using an RNA polymerase gene (*rpoB*) (Lim *et al*., 2003), have failed to reveal the occurrence of virulence genes in various strains (Fauchere, 1996; Kindermann *et al*., 2000; Lamouliatte *et al*., 1996). Attempts have also been made to develop methods for obtaining *H. pylori* genotype data directly from biopsy samples, these methods require extraction of genomic DNA for performing PCR assay (Park *et al*., 2003). Methods reported by Chattopadhyay *et al*. (2004) and Bolek *et al*. (2007), which are mainly based on boiling of biopsy samples and centrifugation followed by PCR assay, are undoubtedly simpler and less time consuming but suffers from reproducibility.

The main objective of this study was to develop a simple and rapid method for the detection and genotyping of *H. pylori* isolates present in patients suffering from numerous types of gastric diseases. The method reported herein does not require cultivation of bacteria, extraction of genomic DNA, boiling of biopsy samples and frequent steps of centrifugation. Using multiplex PCR assay, the prevalence of type I and/ or type II strains, *vacA* signal sequence (s1 and s2), and mid-region (m1 and m2) alleles as well as the presence or absence of a *cagA* gene can be detected in a single reaction, directly from gastric biopsy samples.

2 Material and methods

2.1 Collection of biopsy samples

Two hundred seventy six patients (189 men and 87 women) with mean age of 47 years (range 15 to 80 years) from the Department of Gastroenterology, S. S. Hospital, Banaras Hindu University, Varanasi, India, were included in this study. Each patient underwent upper gastroendocopy for both visual examination and biopsy collection. Three biopsy samples from the antral part of the stomach of each patient were taken using endoscope (CV-70 Videoscope, Olympus, Japan). The biopsy samples were taken from the patients suffering from ulcers (n=92), gastritis (n=60), and/or gastric cancer including both malignant and nodular types (n= 62) and healthy cases (n= 62). Case history of the patients revealed that none had received non steroidal anti inflammatory drugs (NSAIDs), proton pump inhibitors (PPI) or antibiotics during last two months. Consent of each patient was obtained before the collection of biopsy. Out of three biopsy samples, one was used for ultra rapid urease test (URUT), the second for detecting *H. pylori* through *in vitro* culture and the third for performing multiplex PCR assay and genotyping of *H. pylori*.

2.2 Bacterial strains

Two strains, namely, *H. pylori* 26695 (ATCC 700392) and *H. pylori* 60190 (ATCC 49503), were used as reference isolate. Both the strains have the genotype s1/m1 and are *cagA*+. *H. pylori* 26695 was provided by Dr. C. M. Habibullah, Centre for Liver Research and Diagnostics, Deccan College of Medical Sciences and Allied Hospitals, Hyderabad, India, and genomic DNA of *H. pylori* 60190 was obtained from Prof. John C. Atherton, Institute of Infection, Immunity and Inflammation, Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2RD, UK.

2.3 Ultra rapid urease test (URUT)

Ultra Rapid Urease Test was performed as described by Thillainayagam *et al.* (1991). Briefly, each biopsy sample (first set) was placed into a sterile Eppendorf tube containing 0.5 ml of a freshly prepared 10% urea solution in deionised water and two drops of 1% phenol red as a pH indicator. A positive result was noted by a change in the

colour of the solution from orange to pink within the first minute. This test indirectly shows the presence of *H. pylori* in the test sample.

2.4 Culture methods and maintenance

2.4.1 Culture vessel

Glasswares used were of either "Corning" or "Borosil" make. Cultures were routinely grown in culture plate (100 mm diameter), each containing 30 ml medium.

2.4.2 Transport media

Sterilized *Brucella* broth (Bectone-Dickinson) supplemented with 7% (vol/vol) fetal bovine calf serum albumin was used for sample collection and transport of biopsy specimen was done in chilled condition from endoscopy room to culture laboratory. The composition of medium is given bellow.

Composition of growth medium

Table 3.1.1 Composition of *Brucella* Agar

Ingredients	**Quantity (g/l)**
Pancreatic digest of casein	10
Peptic digest of animal tissue	10
Yeast extract	2
Dextrose	1
Sodium chloride	5
Sodium bisulphite	0.1
Agar-agar[a]	15

pH - 7.0 ± 0.2 at 25 ^{o}C adjusted after autoclaving

[a]Agar-agar was used in solid medium only

2.4.3 Sterilization

Culture media and glasswares were sterilized by autoclaving at 121°C and 15 lb/inch^2 pressure for 15 min. Organic chemicals, antibiotics, metabolic inhibitors and other biochemicals were sterilized by membrane filtration through Millipore filters of 0.45 μm pore size (Millipore Intertech Inc., Bedford, MA, USA).

2.4.4 Isolation, growth, maintenance and routine culture of *H. pylori*

Brucella Agar (Table 3.1.1) supplemented with 7% (vol/vol) lysed sheep blood was used as primary isolation medium as well as in routine culture. To avoid contamination and enrichment of *H. pylori* antibiotics namely, 10 mg/l vancomycin, 5 mg/l trimethoprim, 2500 IU/l polymyxin B, and 5 mg/l amphotericin B were added in the culture medium. Freshly collected biopsy samples of different patients were placed on agar plates and gently smeared. Plates were incubated at 37°C, 100% humidity and under microaerophilic conditions (10% CO_2, 5% O_2, 85% N_2) for three to five days. Putative colonies of *H. pylori* appearing after 3-5 days of growth were picked up and streaked on fresh plates. After 2-3 sub-culturing, all the isolates of *H. pylori* were grown on *Brucella* agar supplemented with 7% (vol/vol) lysed sheep blood. Tentative identification of *H. pylori* was made on the basis of colony morphology (small and translucent colonies), shape (curved gram-negative) and positive tests for urease, catalase, and oxidase.

2.4.5 Preservation of *H. pylori* isolates

1 ml of 1% peptone water (Difco) with 25% glycerol was inoculated with a fresh culture of *H. pylori* and stored at -70°C till further use.

2.5 Identification of *H. pylori* by amplification of 16S rDNA by species specific primer

Genomic DNA was extracted employing DNeasy Tissue Kit (Qiagen, GmbH, Hilden, Germany) as per the instructions of manufacturer. 16S rDNA (534-bp) of laboratory grown pure cultures was amplified using *H. pylori* specific primer (Table 3.1.2). Amplification was performed in a PTC-100 Thermal Cycler (MJ Research, Inc., Walthon, MA, USA). The PCR reaction mix included 1.5 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), 1 X PCR buffer with 1.5 mM $MgCl_2$ (1 mM $MgCl_2$ was added additionally so as to attain a final concentration of 2.5 mM), 25 pmole each of

the forward and reverse primers (Integrated DNA Technologies, Inc., Coralville, IA, USA), 0.125 mM each of the dNTPs and 50 ng of template DNA in a total volume of 50 µl. Thermal cycles for the amplification were set at; initial denaturation for 5 min at 94 ^{o}C , 40 cycles of 30 sec at 94^{o}C, 30 sec at 56 ^{o}C and 1 min at 72 ^{o}C followed by a final extension of 5 min at 72^{o}C. The amplified product was visualized on 1.2% agarose gel using a gel documentation unit (BioRad Laboratories, Hercules, CA, USA).

Table 3.1.2 Primers used for amplification of *H. pylori* genes

Gene	Primer	Primer Ssequence (5'-3')	Product Size (bp)	Refrences
16S rDNA	16S rRNA-F	TAAGAGATCAGCCTATGTCC	534	Tiwari *et al*.,
	16S rRNA-R	TCCCACGCTTTAAGCGCAAT		2005
vacA s1/	VAI-F	ATGGAAATACAACAAACACAC	259/286	Atherton *et al*.,
vacA s2	VAI-R	CTGCTTGAATGCGCCAAAC		1995
vacA m1/	VAG-F	CAATCTGTCCAATCAAGCGAG	567/642	Chattopadhyay
vacA m2	VAG-R	GCGTCAAAATAATTCCAAGG		*et al*., 2004
cagA	*cag*5c-F	GTTGATAACGCTGTCGCTTC	350	Chattopadhyay
	*cag*3c-R	GGGTTGTATGATATTTTCCATAA		*et al*., 2004

2.6 Multiplex PCR assay from biopsy samples

The third biopsy sample of each patient was transferred to a microcentrifuge tube containing 120 µl of sterile phosphate buffer saline (PBS) and used for multiplex PCR assay. All the tubes were vortexed vigorously for 2 min, which resulted into the formation of a turbid milky suspension. After the disappearance of milky appearance (which normally occurred within 1-2 min), the resulting transparent solution showed the presence of few small pieces of biopsy tissue, which were carefully removed with sterile forceps. The steps adopted above caused release of bacteria from biopsy tissue to the PBS solution. Three µl of resulting suspension (PBS suspension) from each sample was taken out and used directly as template for multiplex PCR. However, with a view to compare the efficiency and sensitivity of the above procedure, 38 biopsy tissues were boiled, and after centrifugation, the suspension was used for multiplex PCR assay. Furthermore, to

rule out the role of PCR inhibitors which may be present in patient's specimen, template DNA was also recovered by DNeasy Tissue Kit (Qiagen) for performing PCR assay. Multiplex PCR assay was performed in a final volume of 25 µl, which contained 10 pmole of the primer (including both forward and reverse, Table 3.1.2) of 16S rRNA, 10 pmole of VAG, 25 pmole of VA1, 10 pmole of *cag*5c-F and *cag*3c-R, 0.25 mM each of the dNTPs, 0.9 U of *Taq* DNA polymerase, and 1.5 mM of $MgCl_2$ in standard PCR assay buffer (Bangalore Genei, Bangalore, India) and 50 ng of template DNA or 3 µl of suspension (obtained from biopsy samples). The thermal program was set as; initial denaturation for 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by the final extension of 10 min at 72°C. 5 µl of the amplified PCR product was electrophoresed on a 2% agarose gel containing 0.2 mg/ml ethidium bromide. The gels were run in Tris-borate-EDTA (TBE) buffer and monitored as described above.

3 Results

3.1 Diagnosis of patients and screening of *H. pylori* in biopsy samples by multiplex PCR

Preliminary clinical test performed by upper gastrointestinal endoscopy revealed that out of 276 patients, 214 had different types of gastrointestinal diseases and 62 was normal. With a view to screen the presence of *H. pylori*, URUT was done with fresh biopsy samples of all the patients. Of the 276 patients, URUT gave a positive test in biopsy of 221.

To confirm the results of URUT, amplification of *H. pylori* specific 16S rDNA and multiplex PCR assay targeting *cagA*, *vacA* and *H. pylori* specific 16S rRNA gene was done. For achieving the above objective, template for the amplification of *H. pylori* specific 16S rDNA and/or multiplex PCR assay from biopsy samples was recovered by a modified method. Briefly, biopsy samples were vortexed, and after removing the small pieces of tissues, the resulting suspension (devoid of tissues) was used as a source of template. Results with the above template showed excellent amplification of *H. pylori* specific 16S rDNA in 226 patients (of 276). Identical result was obtained when multiplex PCR assay was performed with the template used above (obtained by vortexing of biopsy tissues from 226 patients). It is pertinent to mention that specimens (50) that did not show

amplification of *H. pylori* specific genes by vortexing method showed negative results even with the template DNA recovered by Qiagen kit suggesting that PCR inhibitors may not be present in the suspension. The typical representation of multiplex PCR showing amplification of *vacA* s1, *vacA* s2, *vacA* m1, and *vacA* m2 alleles , *cag A*, and 16S rDNA from seven different biopsy samples is shown in the agarose gel photograph (Fig. 3.1.1). The amplified products were comparable to those obtained by pure culture of *H. pylori* (Fig. 3.1.2). That the amplified products are indeed originating from *H .pylori* were evident from the size and profile of different amplicons as observed in the reference isolate (Fig. 3.1.2). Interestingly, the presence of *H. pylori* by multiplex PCR assay was detected in 28 subjects that otherwise, did not show any sign of gastrointestinal diseases.

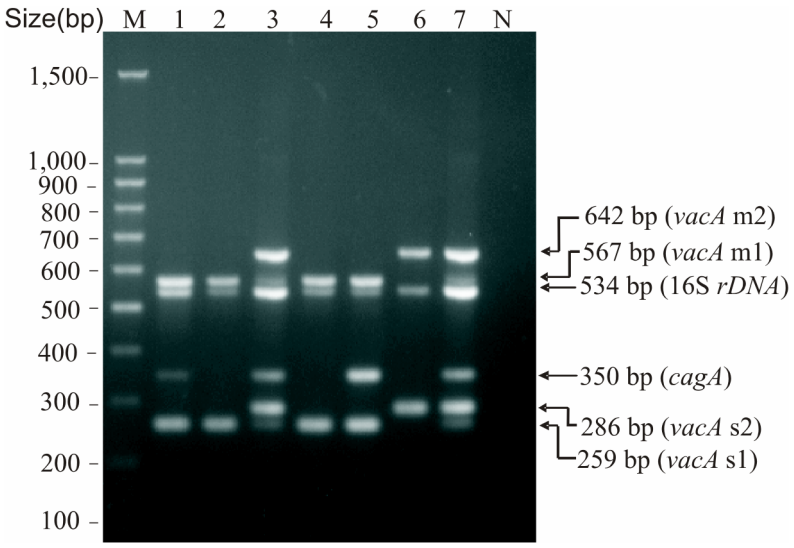


Fig. 3.1.1 Representative multiplex PCR for the amplification of *vacA* s1, *vacA* s2, *vacA* m1, and *vacA* m2 alleles, *cagA* gene, and 16S rDNA of *H. pylori* from biopsy of 7 patients. Fresh biopsy samples obtained from patients admitted to S.S. Hospital, Banaras Hindu University, were directly used in PCR assay. Equal amount of amplified product was loaded into each well. Arrow on the right-hand margin denotes the location of various genotypes. Lane M, 100-bp marker (Promega, Madison, WI, USA); lane 1, ATCC 26695, s1/m1 *cagA* 16S rRNA; lane 2, s1/m1 16S rRNA; lane 3, s1/s2 m1/m2 *cagA* 16S rRNA; lane 4, s1/m1 16S rRNA; lane 5, s1/m1 *cagA* 16S rRNA; lane 6, s2/m2 16S rRNA; and lane 7, s1/s2 m1/m2 *cagA* genotype. Lanes 3 and 7 show multiple strain infection, and lane N shows negative control (without template). Template from the remaining 219 biopsy samples showed amplifications of various genes depending upon their respective genotypic constitution.

Once it became evident that the vortexing method could be routinely used for the detection purpose, it was desirable to test the sensitivity of this method. Accordingly, template DNA from 38 different biopsy samples was obtained by boiling as well as vortexing methods for the amplification of desired amplicons with a view to ensure the sensitivity of the protocols used. Results showed that boiling of biopsy samples could not show amplification of desired amplicon in 2 samples (>5 %) whereas vortexing method resulted into amplification of desired genes from all the 38 biopsy samples (100%).This experiment was repeated several times with different biopsy samples and the results obtained were always reproducible. In addition, the intensity of the desired amplicons in majority of the samples was relatively poor in boiling method in comparison to vortexing method. It is thus apparent that the steps required for DNA extraction from culture or biopsy samples could be avoided to perform PCR assay in the vortexing method.

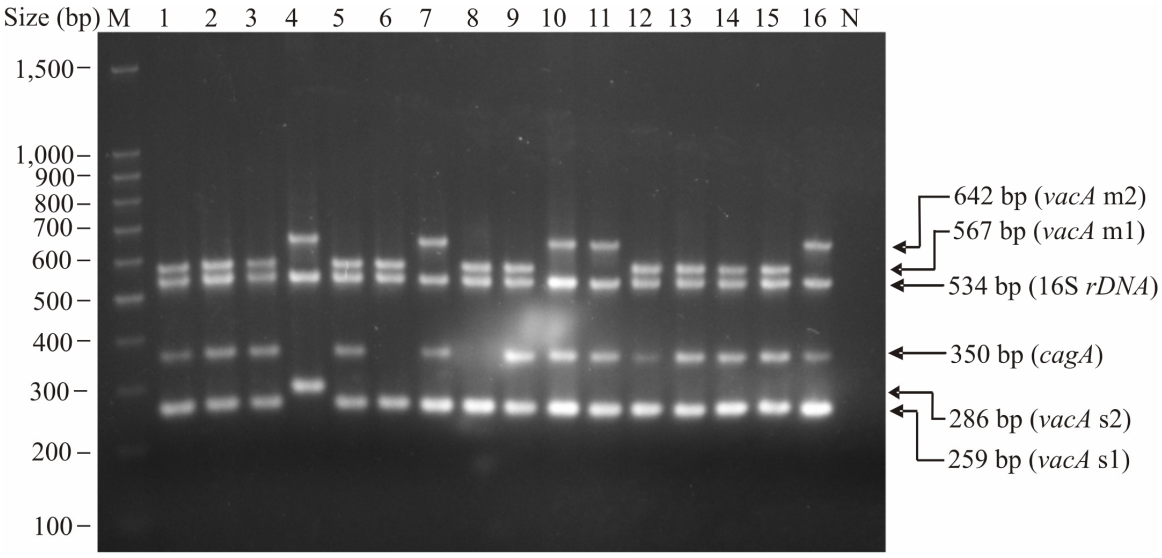


Fig. 3.1.2 Amplification of *vacA* s1, *vacA* s2, *vacA* m1 and *vacA* m2 alleles, *cagA* gene, and 16S rDNA of pure culture of *H. pylori* by multiplex PCR. DNA was extracted from laboratory-grown cultures. Arrow on the right-hand margin denotes the location of various amplified products in different isolates. Lane M, 100-bp marker (Promega); lane 1, ATCC 26695, s1m1 *cagA* 16S rRNA; lane 2, ATCC 60190, s1m1 *cagA* 16S rRNA; lanes 3, 5, 9, and 12 to 15, s1m1 *cagA* 16S rRNA; lane 4, s2m2 16S rRNA; lanes 6 and 8, s1m1 16S rRNA; lanes 7, 10, 11, and 16, s1m2 *cagA* 16S rRNA; and lane N, negative control (without template).

Further analysis of data based on both URUT and PCR assay showed the presence of *H. pylori* in 217 patients and their absence in 46 patients (n= 263 of 276). In the remaining 13 samples, 9 was positive with PCR assay and 4 with URUT only. Evidently, our results showed a sensitivity of 96% and specificity of 92% when the results of multiplex PCR assay were compared with URUT. Of the 226 *H. pylori* positive biopsy samples, we could successfully isolate and grow culture of *H. pylori* from 198 samples. Notably, multiplex PCR assay performed with biopsy isolates showed amplification of desired amplicons of *H. pylori* in all the 198 samples.

3.2 Test for the prevalence of *H. pylori*

Among the 276 patients, *H. pylori* was detected in 226 (81.88%) comprising of 54 gastritis, 86 ulcer, 52 cancer and 34 normal cases. Further analysis revealed that out of 276 patients, 36 had multiple *H. pylori* strain infection and 8 (5 gastritis patients and 3 normal cases) showed the amplification of 16S rDNA only (*cagA*$^-$ and *vacA*$^-$). Unless otherwise stated 232 patients were chosen for further study (Table 3.1.3). Genotypic profile of *H. pylori* from all the 182 patients (excluding 50 *H .pylori* negative patients) is presented in Table 3.1.3. Form the data it is evident that the prevalence of *H. pylori* was highest, 70 (92.1%) in patients suffering from ulcer followed by, 40 (86.9%) in gastritis. The number was 44 (81.5%) in cancer patients and 28 (50%) in normal cases (Table 3.1.3).

3.3 Genotyping and gene combinations

After confirming the presence of *H .pylori* in different patients, genotypic analysis of *vacA* and *cagA* genes was carried out. Of the 182 patients suffering from single strain infection, s1 genotype of *vacA* was detected in all the 40 (100%) gastritis and 44 (100%) cancer patients, whereas the number was 61 (87.1%) in ulcer and 23 (82.1%) in normal patients (Table 3.1.3). Altogether, 168 (92.3%) patients had s1 genotype. Interestingly, the s2 genotype was noted in 9 (12.9%) ulcer and 5 (17.9%) normal patients. The *vacA* m1 genotype was detected in 106 (58.2%) patients, the highest percentage (80.0%) being in gastritis followed by ulcer (60.0 %) patients. *vacA*m2 genotype was highest in normal (75.0%) followed by cancer patients (43.2%) (Table 3.1.3).

Further analysis of *vacA* revealed that s1/m1 combination was more common owing to the fact that 106 (58.2%) patients carried this genotype, while s1/m2 genotype was

present in 62 (34.1%) patients (Table 3.1.4). Notably, the frequency of s2/m2 genotype was smaller; only 14 (7.7%) patients had this combination. As expected, s2/m1 genotype was not present in any patient. Results obtained by multiplex PCR assay also showed that of 276 patients, 36 patients had mixed infections and exhibited different combination of both s1 and s2 from the signal region and m1 and m2 from the middle region (Table 3.1.5). Altogether, our results clearly demonstrate the possible occurrence of multiple *H. pylori* strain infection in different patients.

Table 3.1.3 Status of *cagA* and *vacA* genes of *H. pylori* from patients with different clinical outcomes

Genotype	Status (+ or -)[a]	Gastritis (n[b] = 46)	Ulcer (n = 76)	Cancer (n = 54)	Normal (n=56)	Total (n = 232)
H. pylori	(+)	40(86.9%)	70(92.1%)	44(81.5%)	28(50.0%)	182(78.5%)
	(-)	6(13.1%)	6(7.9%)	10(18.5%)	28(50.0%)	50(21.5%)
cagA	(+)	28(70.0%)	38(54.3%)	44(100%)	5(17.9%)	115(63.2%)
	(-)	12(30.0%)	32(45.7%)	0(0%)	23(82.1%)	67(36.8%)
vacA s1	(+)	40(100%)	61(87.1%)	44(100%)	23(82.1%)	168(92.3%)
	(-)	0(0%)	9(12.9%)	0(0%)	5(17.9%)	14(7.7%)
vacA s2	(+)	0(0%)	9(12.9%)	0(0%)	5(17.9%)	14(7.7%)
	(-)	40(100%)	61(87.1%)	44(100%)	23(82.1%)	168(92.3%)
vacA m1	(+)	32(80.0%)	42(60.0%)	25(56.8%)	7(25.0%)	106(58.2%)
	(-)	8(20.0%)	28(40.0%)	19(43.2%)	21(75.0%)	76(41.7%)
vacA m2	(+)	8(20.0%)	28(40.0%)	19(43.2%)	21(75.0%)	76(41.7%)
	(-)	32(80.0%)	42(60.0%)	25(56.8%)	7(25.0%)	106(58.2%)

[a]+ Present and, - Absent.
[b]Number of patients.

Table 3.1.4 Occurrence of *vacA* genotypes and *cagA* gene of *H. pylori* in patients suffering from gastrointestinal disease

vacA Genotype	Gastritis (n[a]=40)		Ulcer (n=70)		Cancer (n=44)		Normal (n=28)	
	cagA^{+b}	*cagA*$^{-}$	*cagA*$^{+}$	*cagA*$^{-}$	*cagA*$^{+}$	*cagA*$^{-}$	*cagA*$^{+}$	*cagA*$^{-}$
s1/m1	24	8	28	14	25	0	5	2
s1/m2	4	4	10	9	19	0	0	16
s2/m2	0	0	0	9	0	0	0	5

[a]Number of *H. pylori* positive patients.
[b] + Present and -, Absent.

Table 3.1.5 Screening of cases showing the presence of *H. pylori* with varying genotypes

Disease	Gene (s) Combination	Number of patients
Gastritis	*s1s2m1m2/cagA-*	9
Ulcer	*s1s2m1m2/cagA+*	7
	s1s2m2/cagA+	3
	s1s2m1m2/cagA-	6
Cancer	*s1s2m1m2/cagA+*	5
	s1m1m2/cagA+	3
Normal	*s1s2m1m2/cagA-*	3

3.4 Relationship between *cagA* gene and *vacA* genotypes

After demonstrating the variations in *vacA* genotypes, we became interested to reveal relationship, if any, between *cagA* gene and *vacA* genotypes. It is evident from our results that of 40 gastritis patients infected with *H. pylori* and having *vacA* s1 genotype, 32 (80%) patients had s1/m1 genotype, of which 24 (75%) was *cagA* positive (Table 3.1.4). Similarly, in ulcer patients, 61 (87.1%) had *vacA* s1 genotype; of which 42 (60%) had s1/m1 genotype, 28 (66.6%) being *cagA* positive (Tables 3.1.3 and 3.1.4). The s2/m2 genotype was present in 9 patients, all being *cagA* negative. In cancer patients, s1/m1 and s1/m2 genotypes were detected in 25 (56.8%) and 19 (43.2%) patients respectively, all

being *cagA* positive (Table 3.1.4). Genotypic analysis of 28 patients who had no sign of gastrointestinal diseases but carried *H. pylori* revealed the presence of s1/m2 genotype in 16 (57.1%), all being *cagA* negative. 7 had s1/m1 genotype in which 5 was *cagA* positive and 2 was *cagA* negative. The remaining 5 contained s2/m2 genotype, and all were *cagA* negative.

4 Discussion

During the last two decades, a number of both invasive and noninvasive diagnostic tests have been reported for the detection and screening of *H. pylori* infection, all these tests differ in sensitivity and specificity and thereby have limited applications in clinical studies (Cardinali *et al*., 2003; Gatti *et al*., 2006; Lim *et al*., 2003; Park *et al*., 2003; Rudi *et al*., 1999). A number of workers have reported the use of multiplex PCR assay directly with fresh biopsy samples for the rapid detection and genotyping of *H. pylori* (Bolek *et al*., 2007; Chattopadhyay *et al*., 2004). However, all these methods require either extraction of DNA or boiling of biopsy samples to obtain template DNA (Bolek *et al*., 2007). In contrast, the method reported in this study does not require boiling or extraction of DNA from biopsy samples. In fact, mild vortexing and removal of small pieces of biopsy tissues from the suspension enable amplifications of 16S rRNA, *cagA*, and *vac*A genes in a single reaction by multiplex PCR assay. Our findings are in accordance with the report of Chattopadhyay *et al*. (2004), where amplification of *vacA* and *cagA* genes was obtained by boiling method. However, the method used by us differs from earlier methods mainly in two ways: a) boiling of biopsy samples is not essential and, b) removal of biopsy tissues is required for routine amplification of 16S rRNA gene or *vacA* and *cagA* genes. Altogether, vortexing method seems superior to boiling method because all the biopsy samples showed excellent amplification of all the desired amplicons. We are routinely using above method; there has been never failure and the results obtained are repeatable. Failure in getting amplification of *H. pylori* specific genes or poor amplification by boiling method as reported by other workers could be due to the release of PCR inhibitors from the biopsy tissues and/or the presence of impurities in the extracted DNA (Bolek *et al*., 2007). It has been reported that clinical specimens frequently contain PCR inhibitors that bind to *Taq* polymerase and inhibits its activity. That there is no release of PCR inhibitors in the suspension after vortexing of biopsy tissue is evident from the fact that the results of PCR assay performed with template DNA

recovered by commercial kit (Qiagen DNeasy Tissue Kit is known to give yield of DNA free of contaminants and enzyme inhibitors) were similar to those obtained by vortexing method for the detection or genotyping of *H. pylori*. Such a conclusion is further supported by another set of experiment where PCR assay was performed with the addition of standard *H. pylori* cells in the suspension (obtained after vortexing) of clinical samples showing negative results. Interestingly all the samples showing negative results showed excellent amplification of *H. pylori*-specific genes, thereby confirming the absence of PCR inhibitors in the vortexed suspension. Apparently, the method adopted by us seems rapid, simple, and convenient because the steps involved herein do not require centrifugation, extraction of DNA, or isolation and culturing of bacteria. In addition, amplification of 16S rRNA gene together with *vacA* and *cagA* genes could be done in a single reaction by multiplex PCR. To date, no report exists for the multiplex PCR assay comprising 16S rRNA, *vacA* and *cagA* genes in a single reaction for the screening and detection of *H. pylori*. Evidently, the results obtained in the present investigation are interesting and represent a step forward in the search for a simple and rapid methodology for detecting *H. pylori* infection in patients suffering from various types of gastrointestinal diseases. PCR assay as such is the most reliable method for the detection of microbes in clinical specimen. The method reported in this study may be useful especially in hospitals where a large number of clinical specimens require screening and detection of bacteria.

The outcome and efficacy of our method (which is mainly based on multiplex PCR assay from the fresh biopsy sample) are also evident from the data of *vacA* genotyping and *cagA* gene analysis of *H. pylori* of 276 patients. Results of the *vacA* genotyping and detection of *cagA* gene in this study are similar to the results of previous reports based on laboratory grown *H. pylori* or DNA extracted from biopsy samples or assay performed directly from the biopsy samples. Similar to earlier reports, we observed that in all the 182 *H. pylori*-positive patients, *vacA* s1 genotype was predominant, the value being 92.3 % covering all types of patients. The percentage of strains with s2 genotype was lower (7.7 %). Furthermore, the combination of s1/m1 was higher (58.2 %) than s1/m2. Validity of genotypic analysis made directly from biopsy samples is supported from the results of *in vitro* culture data. We have clearly demonstrated that all the laboratory-grown isolates (198) also showed the dominance of s1/m1 genotype thereby confirming the results of biopsy samples. Almost similar findings pertaining to

genotyping of *H. pylori* strains were obtained from Calcutta, West Bengal, by Mukhopadhyay *et al*. (2000), and thus, our results are in agreement with their findings. Most probably, the strains of *H. pylori* reported in this study could be similar at least in terms of s1/m1 combination with those reported from the patients of Calcutta (Mukhopadhyay *et al*., 2000). From our results, it may be inferred that strains reported from Calcutta patients are in no way distinct, and possibly, all the strains of *H. pylori* from North-East India might be having identical genotype consisting of *vacA* s1 or *vacA* s1/m1 and may be *cagA* positive. This is possible because the geographical location of Kolkata and Varanasi is apparently not very diverse and living standard of majority of the populations is more or less similar.

Notable differences in the distribution of *cagA* gene in *H. pylori* strains have been reported from different parts of the world (Covacci *et al*., 1999; Matteo *et al*., 2007; Mukhopadhyay *et al*., 2000; Xiang *et al*., 1995). It has been demonstrated that *cagA* gene is present in 50 to 70% of *H. pylori* strains in Western populations, whereas it is present in > 90% of strain from Eastern populations (Bolek *et al*., 2007). In this study, 63.2% patients showed *cagA* gene out of 232 cases (including 56 normal). Interestingly all the cancer patients were *cagA* positive, having either s1/m1 or s1/m2 combinations. Notably, the role of *cagA* gene seems critical in gastric cancer in the light of report that *cagA* is the key virulence factor in *H. pylori* strains responsible for causing cancer (Mahboob *et al*., 2005). Several workers have reported relationship between infection with $cagA^{+}$ *H. pylori* strains and a higher risk of gastric cancer. From our results, it may be concluded that patients having infection with *cagA* and *vacA* positive strains of *H. pylori* might have a higher chance of secondary carcinoma. It is also pertinent to mention that *vacA* s1 emerged as the dominant factor in gastritis being present in 100% patients. Owing to the fact that the percentage of *cagA* is significantly higher in gastritis patients in comparison with the ulcer patients, gastritis may be directly linked to s1 allele and *cagA* gene. Variations in *vacA* genotypes demonstrate the prevalence of multiple strain infection in various types of patients. Similar findings were noted in other investigation (Chattopadhyay *et al*., 2004). Altogether, determination of *vacA* genotypes and *cagA* gene may contribute to the clinical identification of patients at different level of risk. Knowing that a difference in sensitivity to therapy between the *H. pylori* genotypes exists, a detailed genotyping seems essential for the treatment of various types of gastrointestinal diseases. Prevalence of *H. pylori* in 28 of 56 normal patients (do not show any clinically

proven outcome of the infection) is no doubt interesting but not surprising. Most probably, *H. pylori* does infect these patients, but virulence may not be manifested. Alternatively, the titre value of *H. pylori* is very low and thereby clinical symptoms are not detectable. We feel that these patients would become clinically infected in due course of time after the active growth of *H. pylori* or by the activation of cascade of genes required for virulence (Ewald, 1994). It would be desirable to make detailed study to ascertain the exact reasons why these patients do not exhibit any symptoms.

In conclusion, multiplex PCR assay seems useful for screening of a large number of clinical specimens. We have demonstrated that patients infected with *vacA*$^+$ and *cagA*$^+$ *H. pylori* strains were more prone to gastric cancer. It is felt that genotyping of *H. pylori* strains might be useful in clinical identification of patients at different level of risks, which in turn may prove beneficial in the treatment of the patients.

It is felt that further study targeting other genes such as *babA* and *iceA* are essential to extend the usefulness of method described above, which is primarily based on culture independent approach but requires fresh biopsy samples. We would recommend that laboratories with moderate facilities may use multiplex PCR assay for rapid and routine detection and genotyping of *H. pylori* using fresh biopsy samples of patients suffering from various types of gastrointestinal diseases.

Section 2

Genetic Diversity

Genetic Diversity among North and South Indian Isolates

1 Introduction

H. pylori is predominantly transmitted within families and infection occurs mostly in early childhood, frequently leading to persistent infection lifelong (Kusters *et al*., 2006). Notably, only a fraction of colonized individuals (Blaser *et al*., 1995; Nomura *et al*., 1991; Taylor and Blaser, 1991) develop clinical sequelae which perhaps depend on factors like host characteristics that are governed by genetic polymorphisms (El-Omar, 2001), differentially represented bacterial determinants and/or the specific interactions between a particular strain and its host that occur during decades of coexistence. Although initially the *H. pylori* population structure appears to be clonal, substantial genetic diversity is seen in isolates obtained from different individuals that are consistent with extensive recombination and a panmictic population structure (Achtman and Suerbaum, 2000; Akopyanz *et al*., 1992; Suerbaum *et al*., 1998; van Doorn *et al*., 1998). Various factors are responsible for genetic diversity among strains which include chromosomal organization (Alm *et al*., 1999; Jiang *et al*., 1996; Taylor *et al*., 1997), point mutations in highly conserved genes (Achtman *et al*., 1999), the presence of nonconserved (Akopyants *et al*., 1998) and/or mosaic forms of genes (Chattopadhyay *et al*., 2004). Phenotypic variations as well as the immediate cellular adaptations to stress are caused by mutations however environmental challenges are resisted by organisms by changing their genetic material (by mutation or horizontal gene transfer). Apparently, *H. pylori* lacks many inducible responses that are present in *E. coli*. For instance, the regulatory genes *oxyR* (oxidative stress), *fnr* (fumarate and nitrate regulation), *crp* (carbon utilization), *rpoH* (heat shock), and *lexA* (DNA damage- induced SOS response) are absent (Tomb *et al*., 1997). It has also been demonstrated that *H. pylori* lacks the rigorous response (Scoarughi *et al*., 1999). This indicates that the regulation of gene expression at transcription level occurs less often in *H. pylori* than in *E. coli* and *H. pylori* prefers to use DNA mutation as a strategy to adapt to changing environments. Development of antibiotic resistance is one of the significant examples of this strategy. In spite of the fact that *H. pylori* can be efficiently transformed with resistance genes from related species *in vitro*, it has been clinically observed that resistance occurs due to mutations of

chromosomal genes, instead of acquiring resistance gene(s) carried on plasmids or transposons (Taylor, 1997). Although host factors that may differ among individuals, seem to play an important role in determining which strain of *H pylori* predominates in animal models, equivalent factors in human beings may also play an important role in susceptibility to initial or persistent infection and for disease.

Several typing methods have been described for *H. pylori* among which; (i) SDS-PAGE of whole cell proteins, (ii) randomly amplified polymorphic DNA (PCR-RAPD) (Owen *et al*., 1994), (iii) repetitive sequence element polymerase chain reaction (REP-PCR), (iv) DNA-DNA hybridization (Yoshimura *et al*., 1993), (v) ribotyping (Morgan and Owen, 1990), (vi) restriction endonuclease analysis of genomic DNA (REA), (vii) PCR-RFLP (restriction fragment length polymorphism) (Akopyanz *et al*., 1992), (viii) whole genome pulsed-field electrophoresis, and (ix) multilocus enzyme electrophoresis (Go *et al*., 1996) are the most common. Genetic heterogeneity of *H. pylori* is confirmed by majority of these methods. Bacterial strains with different pathogenicity are often differentiated by genotypic typing methods. The prospect of the disease can be predicted by correlation between a certain genotype and clinical symptoms. It has been demonstrated by Covacci *et al*. (1993) and Xiang *et al*. (1993) that phenotypic expression of an immunodominant toxin-associated protein (CagA) and a vacuolating toxin (VacA) is seen more frequently in *H. pylori* strains isolated from patients with duodenal ulcers than from patients with gastritis. Although assumptions have been made that genetic variation provides *H. pylori* with a means to adapt to the individual host organism, thereby contributing to its ability to cause persistent infection, there is very limited evidence to support this, and a more systematic analysis of the impact of genetic diversity during chronic infection on pathogen fitness is required. Gastrointestinal diseases are very common among Indians. Unfortunately little, if any, work has been conducted on the prevalence and genetic variations among the most notorious pathogeneic bacterium, *H. pylori* especially in North Indian isolates.

2 Material and methods

2.1 Patients and bacterial strains

Thirty six *H. pylori* strains comprising fourteen from 14 patients of Varanasi (North India) and twenty two from 22 Hyderabad (South India) patients were isolated from the gastric biopsy samples. All these patients (20 men and 16 female) had mean age of 45 years. Each patient underwent upper gastroendocopy for both visual examination and biopsy collection. Biopsies from the antral part of the stomach of each patient were taken using endoscope. The biopsy samples were taken from the patients suffering from ulcer, gastritis and/or gastric cancer including both malignant and nodular types and healthy cases. Case history of the patients and other details including source of reference isolates are described in section I.

2.2 Preparation of PCR products for sequencing and identification

16 rDNA (534 bp) amplified by *H. pylori* specific primer (see section I Table 3.1.2) was purified by Invitrogen Kit (Invitrogen Corpn, CA, USA) following the instructions of manufacturer. The PCR product was eluted in Milli Q water and purity and concentration of DNA were checked by reading OD at 230, 260 and 280nm. The sequencing PCR reaction mix (DNA sequencing kit, Applied Biosystems, Foster city, CA, USA) included 8.0 µl BigDye Terminator V3.0 cycle sequencing ready reaction mixture with Ampli*Taq* DNA polymerase,10 ng PCR product DNA, 3.2 pmole forward primer and deionised water added to achieve total volume of 20 µl. The thermal cycle conditions were set as; initial denaturation at 98^{o}C for 1 min, 25 cycles of denaturation at 96 ^{o}C for 10 s, annealing at 55 ^{o}C for 5 s and extension at 60 ^{o}C for 4 min and hold at 4 ^{o}C until purification steps. Ethanol precipitation was done for the removal of unused BigDye Terminator present in the reaction mixture. After drying the sample, 20 µl of TSR (template suppression reagent) was added, mixed well and heated for 2 min at 95 ^{o}C. Subsequently the samples were chilled on ice and after vortexing thoroughly, centrifuged briefly in a microcentrifuge. Samples were hold in ice until loading. Sequencing was performed in an ABI-PRISM 310 Genetic Analyzer (Applied Biosystems). *cagA*, *cagE*, *cagT*, *vacA*, *iceA*1 and *iceA*2 genes of selected isolates were also sequenced by similar method but annealing temperature of *cagE*, *cagT*, *iceA*1 and *iceA*2 was set at 52 ^{o}C. The primers of sequencing PCR were same as used for normal or multiplex PCR of respective

genes. PCR and direct sequencing were performed at least twice to determine and confirm the DNA sequences for each isolate. All the sequences were matched against nucleotide sequences present in Gene Bank using the BLASTn program (Altschul *et al*., 1997) at website www.ncbi.nlm.nih.gov/blast.

2.3 Amplification of full length 16S rDNA

16S rDNA (1.5 kb) was amplified using universal primer. Amplification was performed in a final volume of 50 µl. The PCR reaction mix included 1.5 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), 1 X PCR buffer with 1.5 mM $MgCl_2$, 300 ng each forward and reverse primers (Integrated DNA Technologies, Inc., IA, USA), each dNTPs at 125 µM (Bangalore Genei) and 50 ng of template DNA. Sequence of primers (8f and 1495r) used for amplification is given in Table 3.2.1 Thermal cycles for the amplification were set as; 4 min at 94^o C, 35 cycles of 1 min at 94^o C, 1 min at 58^o C and 2 min at 72^o C followed by final extension for 5 min at 72^o C and storage at 4^{o}C.

Table 3.2.1 Primers used for diversity analysis of *H. pylori*

Genes	Primers	Sequences (5'-3')	References
16S rRNA	8f	AGAGTT TGATYMTGGCTCAG	Verma *et al*., 2004
	1495r	CTACGGCTACCTTGTTACGA	
ERIC	ERIC-1F	ATGTAAGCTCCTGGGGATTCAC	Hussain *et al*., 2004
	ERIC-2R	AAG TAA GTGACT GGG GTG AGC G	
AFLP	HI-**A**	GGTATGCGACAGAGCTT**A**	Gibson *et al*., 1998
	HI-**T**	GGTATGCGACAGAGCTT**T**	
	HI-**C**	GGTATGCGACAGAGCTT**C**	
	HI-**G**	GGTATGCGACAGAGCTT**G**	
*iceA*1	*iceA*1F	TATTTCTGGAACTTGCGCAACCTGAT	Mukhopadhyay *et al*., 2000
	M.Hpy1R	GGCCTACAACCGCATGCATAT	
*iceA*2	cysSF	CGGCTGTAGGCACTAAAGCTA	Mukhopadhyay *et al*., 2000
	*iceA*2R	TCAATCCTATGTGAAACAATGATCGTT	

2.4 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

16S rDNA (1.5 kb) amplified by PCR was subjected to restriction digestion by *Alu*I and *Rsa*I restriction enzymes as per the instructions of manufacturer (New England Biolabs Ltd, Hertfordshire, UK). Restriction digestion was done in a final volume of 25 µl containing 1 X restriction enzyme buffer, 0.125 µl (1.25 U) restriction enzyme and 15 µl PCR product. After mixing the reaction mixture carefully, samples were incubated for 3 h in a water-bath preset to 37^{o}C. Reaction was terminated by heat inactivation at 70^{o}C for 20 min. The samples were analyzed by agarose (3%) gel electrophoresis and monitored on gel documentation unit (Bio-Rad Laboratories, Hercules, CA, USA).

2.5 ERIC (Enterobacterial Repetitive Intergeneric Consensus) PCR

ERIC- PCR was carried out in 40 µl reaction mix containing; 1 X *Taq* DNA polymerase assay buffer, 125 µM each of dNTPs, 250 ng of each primer (ERIC-1 and ERIC-2) (Table 3.2.1), 1 U *Taq* DNA polymerase (Bangalore Genie) and 50 ng of template DNA. Amplification of inter-rep elements was done using thermal program set as; initial denaturation at 95^{o}C for 3 min, followed by 35 cycles of denaturation at 94^{o}C for 1 min, annealing at 52^{o}C for 1 min and extension at 72^{o}C for 1 min 30s and a final extension at 72^{o}C for 5 min. The reaction mixture was stored at 4^{o}C until use. The amplified products were analyzed by agarose gel electrophoresis (1.5%) as described earlier.

ERIC-PCR pattern of different bacterial isolates was analyzed and used for the construction of dendrogram with the help of Quantity One® 1-D Analysis Software, version 4.4 (Bio-Rad Laboratories, USA). Similarities between each pair of isolates were estimated from the proportion of homology in banding pattern of ERIC-PCR. Dendrograms were constructed from the similarity matrix by the UPGMA (Unweighted Pair Group Method with Arithmetic averages) method.

2.6 Amplified fragment length polymorphism (AFLP)

An aliquot containing 1 µg of genomic DNA of various *H. pylori* isolates was subjected to restriction digestion by *Hin*dIII restriction enzyme as per the instructions of manufacturer (Promega, USA). Restriction digestion of genomic DNA was performed in a final volume of 20 µl containing 20U enzyme and 1 X restriction enzyme buffer. After

mixing the reaction mixture carefully, samples were incubated at 37°C for 16 h. 5 µl aliquot containing approximately 250 ng of digested DNA was used in a ligation reaction containing 30 pmole of each adapter oligonucleotide namely, ADH1- 5'-ACGGTATGCGACAG-3' and ADH2- 5'-AGCTCTGTCGCATACCGTGAG-3' (Integrated DNA Technologies), 1 U of T4 DNA ligase (Promega) in a final volume of 20 µl and incubated at 37°C for 3 to 4 h as per the instructions of manufacturer. Combined restriction-ligation reaction involved the digestion of 250 ng of DNA with 5 U of *Hind*III in the ligase buffer and reaction mixture as described above. The ligated DNA was precipitated with sodium acetate (final concentration, 2.5 M) and 200 µl of chilled absolute ethanol. The samples were kept at -20°C for 30 min and then centrifuged at 12,000 X *g* for 15 min at 4°C. The precipitated DNA was washed in 70% (vol/vol) ethanol and resuspended in 20 µl of distilled water. 2 µl aliquot was used as template for PCR.

2.7 PCR primers and PCR for AFLP analysis

Four primers namely HI-**A**, HI-**T**, HI-**C** and HI-**G** (Integrated DNA Technologies, Inc) (Table 3.2.1) were used in the PCR for AFLP. Amplification was performed in a total volume of 25 µl containing 2 µl of template DNA, 1.5 mM $MgCl_2$, 25 pmole of each individual primer and 1.0 µl (3 U/ µl) of *Taq* DNA polymerase (Bangalore Genie) and 1 X PCR buffer. The thermal cycles were set as; an initial denaturing step of 94°C for 4 min, 33 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2.5 min. Amplified fragments were separated by electrophoresis in 1.5% (wt/vol) agarose gel in TAE buffer and stained with ethidium bromide (0.2 mg/ml).

2.8 Multiplex PCR for *cagA* and *vacA* genes

Multiplex PCR assay was performed in a final volume of 25 µl which contained 10 pmole primer (see Table 3.1.2) of VAG, 25 pmole of VA1, 10 pmole of *cag*5c and *cag*3c, 0.25 mM each of the dNTPs, 0.9 U of *Taq* DNA polymerase and 1.5 mM of $MgCl_2$ in a standard PCR assay buffer (Bangalore Genei) and 50 ng of template DNA. Thermal cycles were set as: initial denaturation for 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by final extension for 10 min at 72°C. 5 µl of the amplified PCR product was electrophoresed on a 2% agarose gel containing 0.2 mg/ml ethidium bromide. The gels were run in Tris-borate-EDTA (TBE) buffer and monitored as described earlier.

2.9 Amplification of *iceA*1 and *iceA*2 genes

PCR for the amplification of *iceA*1 and *iceA*2 genes was performed in a total volume of 25 µl which contained 25 pmole of each (forward and reverse) primer (Table 3.2.1) for *iceA*1 and *iceA*2, 0.25 mM each of the dNTPs, 0.75 U of *Taq* DNA polymerase and 1.5 mM of $MgCl_2$ in a standard PCR assay buffer (Bangalore Genei) and 100 ng of template DNA. Thermal cycle was set at; initial denaturation for 5 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C, followed by final extension of 5 min at 72°C. 5 µl of the amplified PCR product was electrophoresed on a 1.5% agarose gel containing 0.2 mg/ml ethidium bromide. The gels were run in Tris-borate-EDTA (TBE) buffer and monitored as described earlier.

2.10 Nucleotide sequence accession numbers

The nucleotide sequences of the partial 16S rDNA of 14 Varanasi and 11 Hyderabad and *vac*A, *cag*A, *ice*A1 and *ice*A2 of selected Varanasi isolates were submitted to NCBI and accession numbers for all the strains have been obtained (Table 3.2.2).

2.11 Construction of phylogenetic trees based on 16S rDNA sequences

16S rDNA sequences of 24 isolates (13 Varanasi and 11 Hyderabad isolates) and 160 sequences of isolates reported from different parts of the world were used to construct phylogenetic trees. Two phylogenetic trees were constructed; one based solely on the sequences obtained in the present study (24), and the other contained 24 Indian (present study) and 160 reference strains. For both the trees multiple alignments of sequenced nucleotides were carried out using ClustalW2 (version 2.0.10). Neighbor-joining trees were constructed in MEGA 4.0 (Tamura *et al*., 2007) using bootstrapping at 1000 bootstrap trials with the two-parameter model of Kimura.

3 Results

3.1 Detection of *H. pylori* in different patients

H. pylori was successfully isolated from the biopsy samples of all the thirty six patients belonging to Varanasi (14) and Hyderabad (22). Identification was made on the basis of morphological characters, biochemical tests and amplification of *H. pylori* specific 16S rDNA. It is evident from the gel photograph (Fig: 3.2.1 AB) that all the

isolates (typical representation by selected isolates) showed amplification of desired fragment of 16S rDNA (534 bp) with *H. pylori* specific primer. Additionally results of upper gastrointestinal endoscopy revealed that out of 36 patients, 31 had different types of gastrointestinal diseases and 5 was normal. Among the 14 patients of Varanasi (North India), 4 had gastritis, 3 gastric cancer (GC), 2 duodenal ulcer (DU), 1 gastric ulcer (GU) and 4 was normal. Among Hyderabad (South India) patients, 13 had DU, 4 GU, 2 GC, 2 gastritis and 1 was normal.

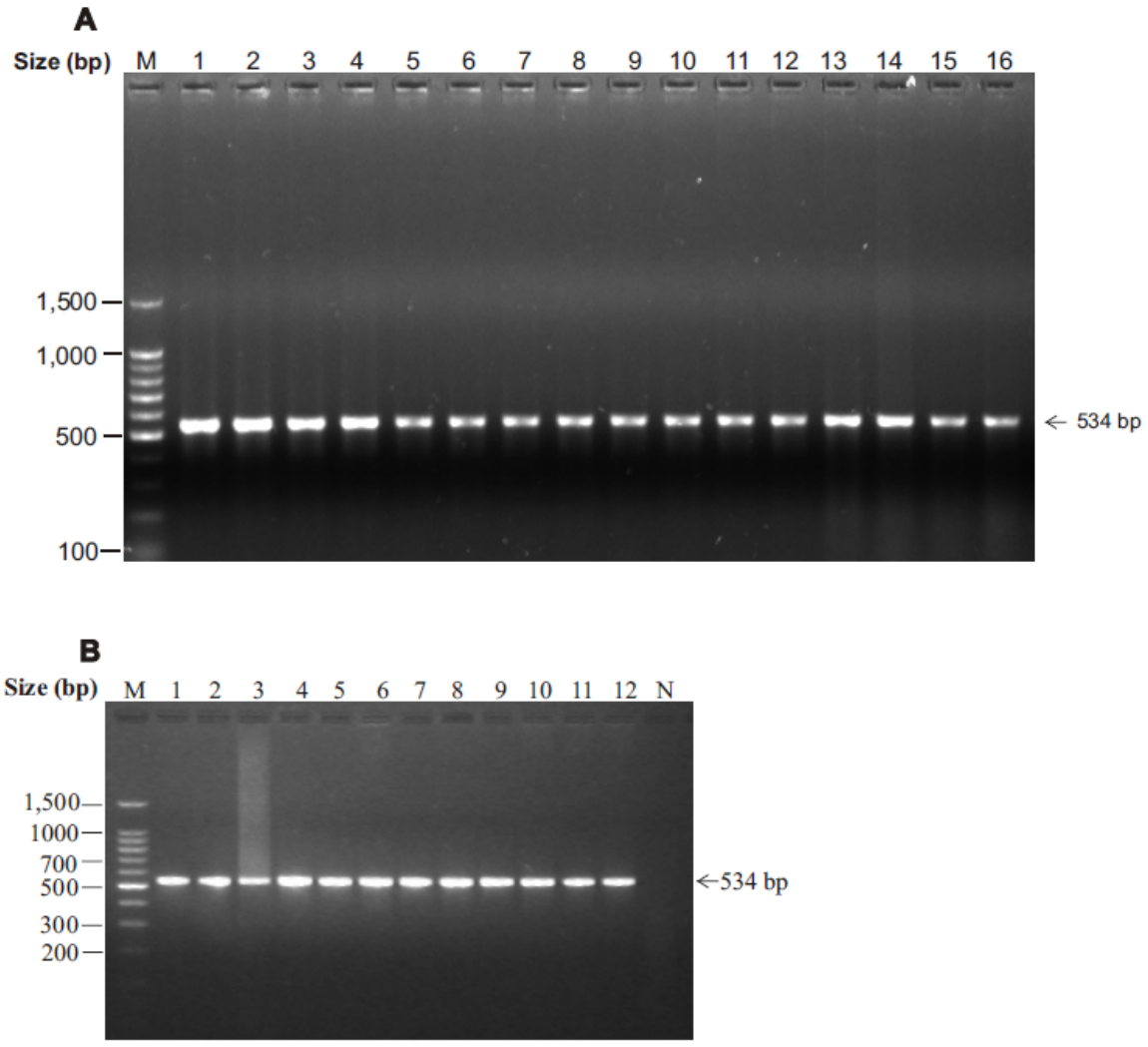


Fig. 3.2.1 Typical representation of 16S rDNA (partial) amplification using *H. pylori* specific primer.

A: Varanasi Isolates; Lanes; M-100bp Ladder, 1-ATCC 26695, 2-ATCC 60190, 3-VAR1, 4-VAR2, 5-VAR3, 6-VAR4, 7-VAR5, 8-VAR6, 9-VAR7, 10-VAR8, 11- VAR9,12-VAR10, 13-VAR11, 14-VAR12,15-VAR13, and 16-VAR14.

B: Hyderabad isolates; Lane M-100bp Ladder, 1-HD1, 2-HD2, 3-HD3, 4-HD4, 5-HD5, 6-HD6, 7-HD7, 8-HD8, 9-HD9, 10-HD10, 11-HD11, 12-HD12, and N- negative control (without template).

3.2 Identification and characterization of *Helicobacter pylori*

As mentioned above amplification of 16S rDNA (534 bp) and pathogenicity genes was achieved in all the isolates but sequencing of 16S rDNA could be done from 11 isolates of Hyderabad and 14 from Varanasi. Additionally sequencing of pathogenicity genes of selected Varanasi isolates was also done (Table 3.2.2). All the sequences were compared with Basic Local Alignment Search Tool (BLAST) BLASTn 2.2.18 in the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) site. Sequence analysis and degree of homology matched with *H. pylori* suggesting that all the 25 isolates are indeed *H. pylori*. Sequences showed 97-99% homology with database available for *H. pylori* at NCBI (Table 3.2.2).

3.3 Diversity based on ARDRA

A typical representation of ARDRA pattern after partial digestion with *Alu*I and *Rsa*I restriction enzymes of 12 isolates belonging to Hyderabad (South India) is shown in Fig. 3.2.2 A, B and C. It is evident from the gel photograph that digestion with *Alu*I shows 3 to 9 bands and on the basis of banding profile all these isolates could be placed in 3 groups (Fig. 3.2.2 B.). The first group included isolates of lanes 1, 2, 3, 4, 5, 6, 8 and 9; second group included- lane 7 and third group lanes 10 to 12. Similarly *Rsa*I gave 3 to 11 bands (Fig. 3.2.2 C) and these bands could be kept in 6 groups (first group; lanes -1, 2, 4, 5, 8 and 9; second group lane- 2; third group lane- 3; fourth group lanes- 6 and 7; fifth group lane- 10 and, sixth group lanes-11 and 12). Further analysis and matching of bands after digestion with both the enzymes suggest that isolates of lanes1, 4, 5, 8 and 9; 2 and 6; 3; 7; 10; 11 and 12 could be identical. Accordingly on the basis of banding profile obtained after *Alu*I and *Rsa*I digestion, all the 12 isolates could be placed in 6 groups. All the remaining isolates of Hyderabad and Varanasi showed almost similar level of diversity.

3.4 ERIC-PCR pattern

Fig 3.2.3 shows a typical representation of ERIC-PCR profile of 12 isolates belonging to Hyderabad. Banding pattern of different fragments clearly reflects diversity among all the isolates. Tentatively these isolates may be kept in 11 groups, only two isolates (lanes 11 and 12) showed homology and could be placed in one group. Degree of diversity in remaining isolates of Hyderabad and Varanasi was in no way different than those observed in 12 isolates (Fig. 3.2.3).

Table 3.2.2 Identification of *H. pylori* by sequencing of 16 rDNA, *vacA*, *cagA*, *cagE*, *cagT*, *iceA*1 and *iceA*2 genes

S. No.	Isolates	Genes/ alleles	Accession No.	Maximum Homology		
				Percentage	Organism	Accession No.
1	VAR1	16S rDNA	FJ200229	98	*H. pylori*	M88157.1
2	VAR2	16S rDNA	FJ200230	98	*H. pylori*	FJ788641.1
3	VAR2	*vacA* s2	FJ984544	97	*H. pylori*	AM088775.1
4	VAR2	*vacA* m2	FJ984545	97	*H. pylori*	AM088768.1
5	VAR2	*iceA*2	FJ984541	97	*H. pylori*	AF176822.1
6	VAR3	16S rDNA	FJ200231	99	*H. pylori*	CP001173.1
7	VAR4	16S rDNA	FJ200232	98	*H. pylori*	FJ788641.1
8	VAR5	16S rDNA	FJ200233	97	*H. pylori*	AY304551.1
9	VAR6	16S rDNA	FJ200234	97	*H. pylori*	FJ788642.1
10	VAR7	16S rDNA	FJ200235	98	*H. pylori*	FJ788641.1
11	VAR8	16S rDNA	FJ200236	98	*H. pylori*	FJ788642.1
12	VAR9	16S rDNA	FJ200237	97	*H. pylori*	FJ788642.1
13	VAR9	*cagE*	FJ984537	99	*H. pylori*	AB191098.1
14	VAR9	*iceA*1	FJ984540	98	*H. pylori*	AF157543.1
15	VAR10	16S rDNA	FJ200238	99	*H. pylori*	FJ788642.1
16	VAR11	16S rDNA	FJ200239	97	*H. pylori*	FJ788641.1
17	VAR12	16S rDNA	FJ200240	99	*H. pylori*	FJ788641.1
18	VAR12	v*acA* m2	FJ984546	97	*H. pylori*	AM088759.1
19	VAR13	16S rDNA	FJ200241	97	*H. pylori*	FJ788641.1
20	VAR13	*cagT*	FJ984539	97	*H. pylori*	AE000511.1
21	VAR13	*vacA* s1	FJ984543	98	*H. pylori*	AB057205.1
22	VAR13	*vacA* m2	FJ984548	96	*H. pylori*	AM088767.1
23	VAR14	16S rDNA	FJ984535	98	*H. pylori*	FJ788642.1
24	VAR14	*cagA*	FJ984536	99	*H. pylori*	AF247651.1
25	VAR14	*cage*	FJ984538	97	*H. pylori*	AB191099.1
26	VAR14	*vacA* s1	FJ984542	99	*H. pylori*	AF217730.1
27	VAR14	*vacA* m1	FJ984547	97	*H. pylori*	AM088759.1
28	HD1	16S rDNA	DQ912962	99	*H. pylori*	M88157.1
29	HD2	16S rDNA	DQ912963	99	*H. pylori*	M88157.1
30	HD3	16S rDNA	DQ912964	98	*H. pylori*	M88157.1
31	HD4	16S rDNA	DQ912965	99	*H. pylori*	M88157.1
32	HD5	16S rDNA	DQ912966	99	*H. pylori*	M88157.1
33	HD6	16S rDNA	DQ912967	99	*H. pylori*	M88157.1
34	HD7	16S rDNA	DQ912968	99	*H. pylori*	M88157.1
35	HD8	16S rDNA	DQ912969	99	*H. pylori*	M88157.1
36	HD9	16S rDNA	DQ912970	99	*H. pylori*	M88157.1
37	HD10	16S rDNA	DQ912971	99	*H. pylori*	M88157.1
38	HD11	16S rDNA	DQ912972	99	*H. pylori*	M88157.1

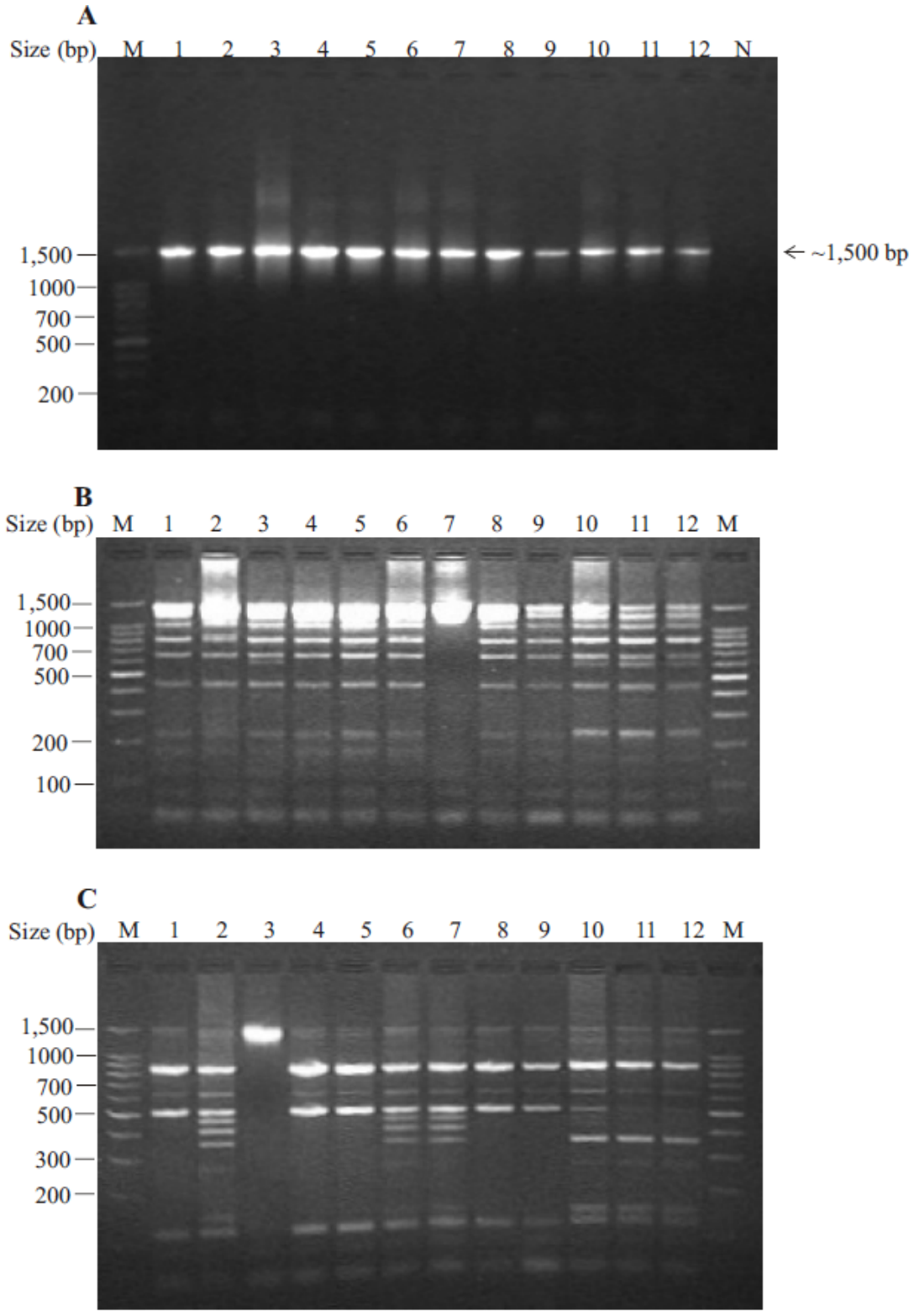


Fig. 3.2.2 Typical representation of 16S rDNA (~1,500bp) amplification and ARDRA profile of Hyderabad isolates.

A: 16S rDNA amplification by universal (eubacteria specific) primer.

B: Partial restriction digestion of 16S rDNA by *Alu*I.

C: Partial restriction digestion of 16S rDNA by *Rsa*I. For A, B and C; Lane M-100bp Ladder, 1-HD1, 2-HD2, 3-HD3, 4-HD4, 5-HD5, 6-HD6, 7-HD7, 8-HD8, 9-HD9, 10-HD10, 11-HD11, 12-HD12 and N- negative control.

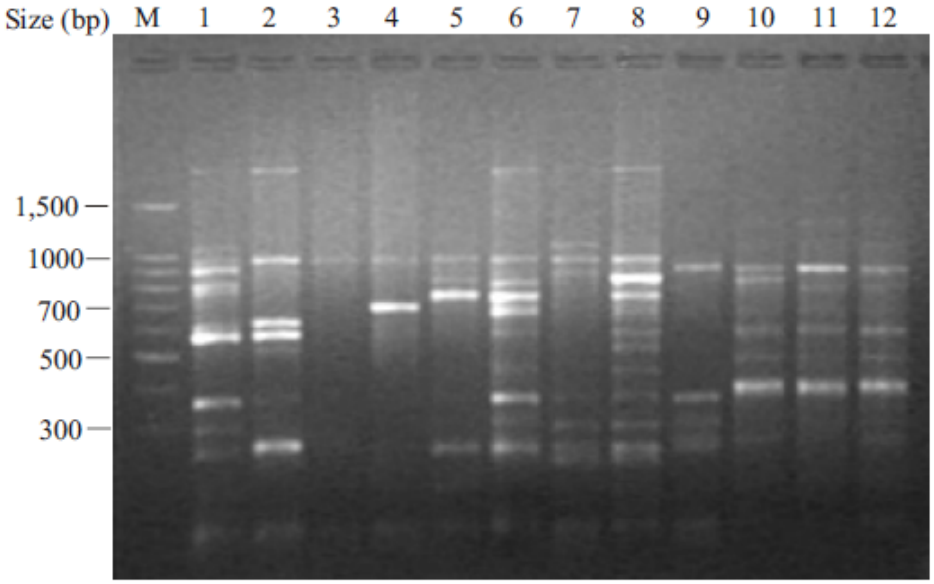


Fig. 3.2.3 Typical representation of ERIC-PCR profile of Hyderabad isolates. Lane M-100bp Ladder,1-HD1, 2-HD2, 3-HD3, 4-HD4, 5-HD5, 6-HD6, 7-HD7, 8-HD8, 9-HD9, 10-HD10, 11-HD11, and 12-HD12.

Dendrogram constructed on the basis of ERIC-PCR profile placed all the 12 isolates in four groups. Group A included five isolates, groups B and D comprised 3 isolates each and there was only one isolate in group C (Fig. 3.2.4). It is evident from the data that group D has 48% similarity with groups A, B and C whereas group C shared 59% similarity with groups A and B. There was 63% homology between groups A and B (Fig. 3.2.4).

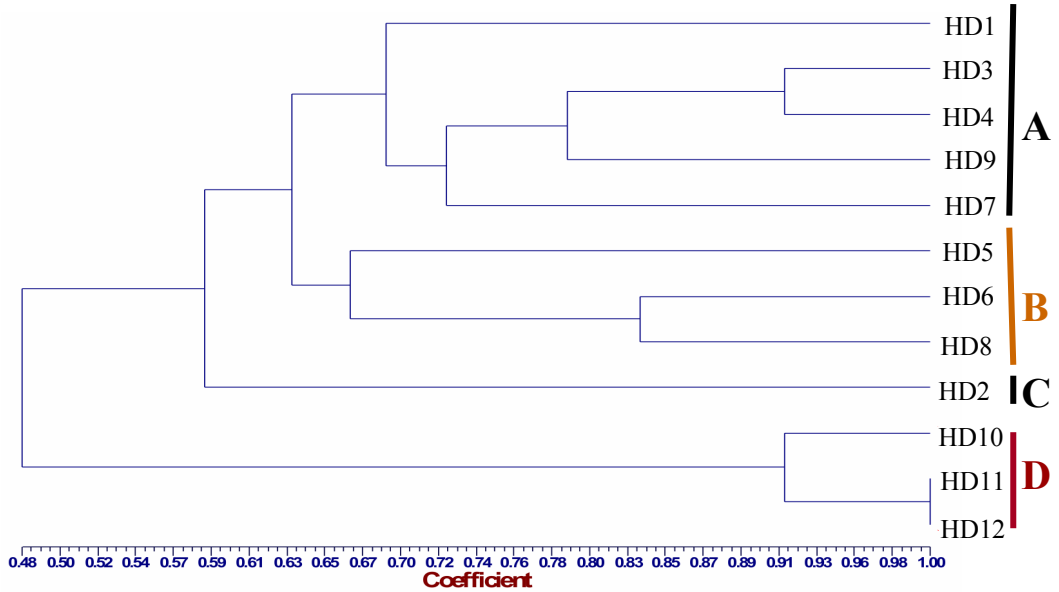


Fig. 3.2.4 Dendrogram constructed on the basis of ERIC-PCR profile for illustrating homology among various isolates.

3.5 AFLP analysis to detect diversity in whole genome

Since 16S rDNA represents a single locus of about 1.5 kb out of the total genome, a phylogenetic analysis on the basis of such a small stretch of DNA would not represent the complete genome. To resolve this problem, we used AFLP for the analysis of whole genome diversity among various strains of *H. pylori*. Accordingly whole genome was digested with hexa-cutting restriction endonuclease *Hind*III followed by ligation with adapter DNA and finally amplified by the primers HI-A and HI-C. It is evident from the typical representation of AFLP of Varanasi and Hyderabad isolates that amplification with HI-A showed 5-12 bands (Fig. 3.2.5) whereas HI-C resulted in 2-8 bands (Fig. 3.2.6; representation by Varanasi isolates). Furthermore it is also apparent from the analysis of banding pattern that all the isolates have distinct banding pattern (Figs. 3.2.5 and 3.2.6).

3.6 Phylogenetic analysis based on AFLP

Knowing that the DNA fingerprinting pattern showed distinct differences among all the 36 isolates, a phylogenetic tree based on the AFLP pattern was constructed. Data of similarity coefficient revealed that all the 38 strains (including two standard ATCC reference strains namely, 60190 and 26695) could be conveniently placed in 3 major groups, namely A, B and C (Fig. 3.2.7). Group A contained 1 strain from Varanasi and 2 from Hyderabad and showed approx. 21% similarity with the strains placed in groups B and C. Group B emerged as an interesting group since it contained 12 strains (10 from Varanasi and 2 reference strain) and shared 30% similarity with the strains of group C. Group C contained 23 strains (3 from Varanasi and 20 from Hyderabad) and formed the largest group. Most importantly none of the isolate showed 100% similarity among each other (Fig. 3.2.7).

3.7 Status of pathogenicity genes

After confirming the presence of *H. pylori* in different patients, genotypic analysis of *vacA*, *cagA*, *iceA*1 and *iceA*2 was carried out (Figs. 3.2.8 and 3.2.9). The study was conducted with 22 isolates from Hyderabad (South India) and 14 from Varanasi (North India). It is evident from the results of multiplex PCR that out of the 22 *H. pylori* isolates from the patients of Hyderabad, s1 genotype of *vacA* was detected in 21 isolates (95.5%) while the s2 genotype was present in 1 (4.5 %) isolate (Table 3.2.3). The *vacA* m1 genotype was present in 8 (36.4%) isolates whereas *vacA* m2 was detected in 14 isolates (63.6%) of different patients. The *cagA* gene was present in 21 (95.5%) isolates. Similarly out of 22 isolates, the *iceA*1 was present in 17 (77.3%) isolates whereas *iceA*2 gene was present in 5 (22.7%) isolates.

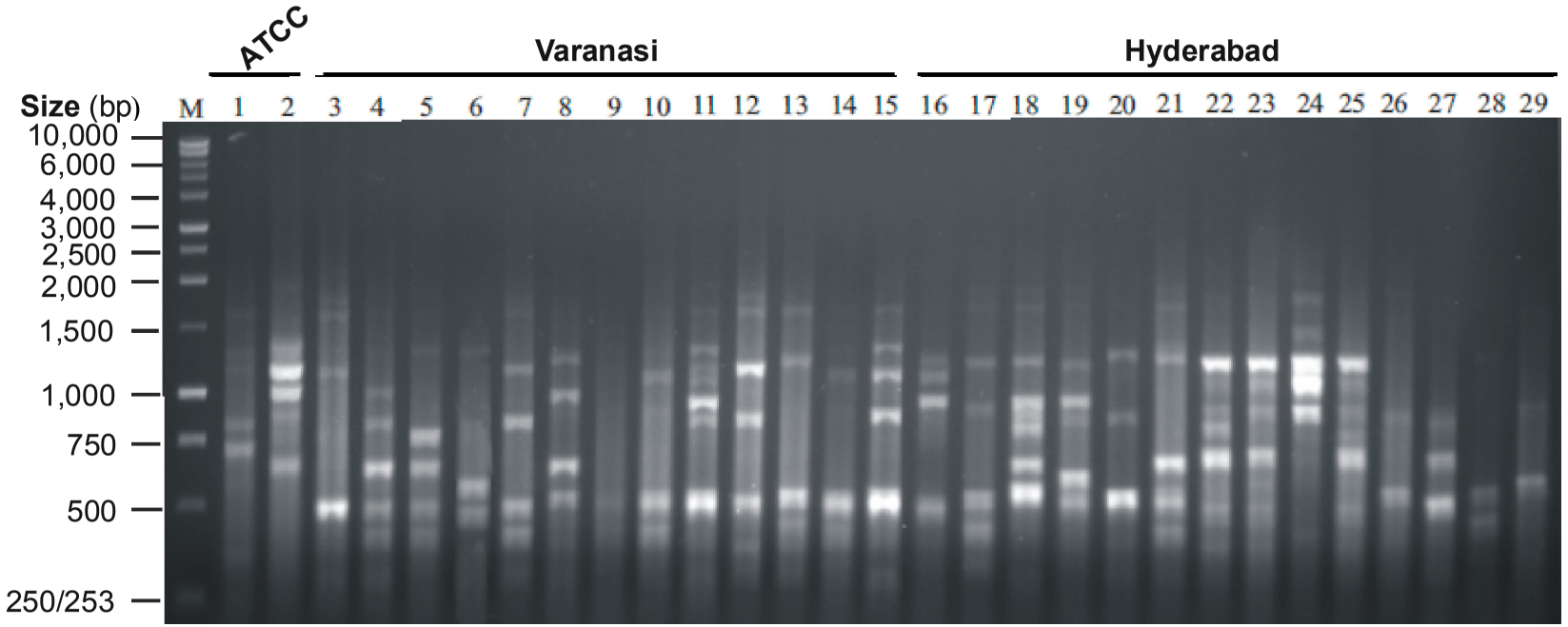


Fig. 3.2.5 Typical representation of AFLP of *H. pylori* isolates. Amplification was done using HI-A. Lane-M 1kb Ladder, 1-ATCC -60190, 2-ATCC-26695, 3-15 Varanasi isolates. Lane 3-VAR1, 4-VAR2, 5-VAR3, 6-VAR4, 7-VAR5, 8-VAR6, 9-VAR7, 10-VAR8, 11-VAR9, 12-VAR10, 13-VAR11, 14-VAR12 and 15-VAR13. Lanes 16-29 Hyderabad isolates; lane 16-HD1, 17-HD2, 18-HD3, 19-HD4, 20-HD5, 21-HD6, 22-HD7, 23-HD8, 24-HD9, 25-HD10, 26-HD11, 27-HD12, 28-HD13, and 29-HD14.

VAR-Varanasi and HD-Hyderabad isolates.

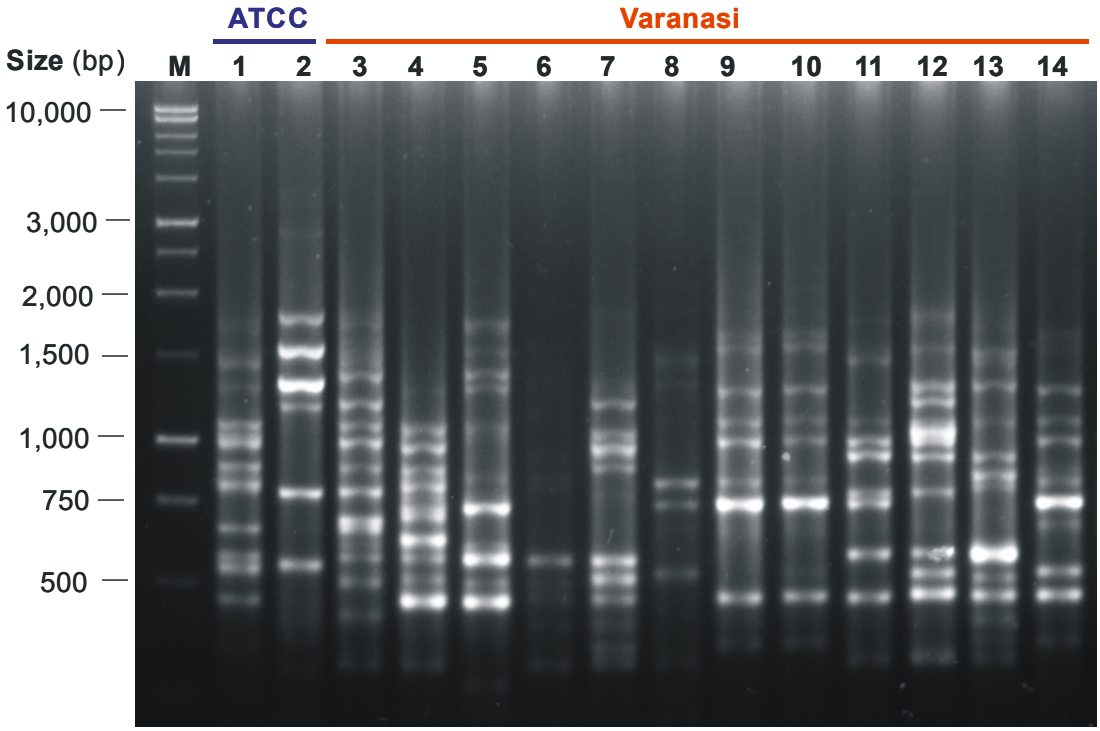


Fig. 3.2.6 Typical representation of AFLP of *H. pylori* isolates following amplification with HI-C. Lane-M 1kb Ladder, 1-ATCC -60190, 2- ATCC-26695, 3-14 Varanasi isolates; Lane 3-VAR1, 4-VAR2, 5-VAR3, 6-VAR4, 7-VAR5, 8-VAR6, 9-VAR7, 10-VAR8, 11-VAR9, 12-VAR10, 13-VAR11, and 14-VAR12.

Analysis of Varanasi isolates (14) revealed the presence of *vacA* s1 genotype in 13 (92.9%) and s2 in one (7.1%) isolate (Table 3.2.4). The *vacA* m1 and m2 genotypes were present in 9 (64.3%) and 5 (35.7%) isolates respectively. The *cagA* gene was detected in 11(78.6%) isolates and *iceA*1 and *iceA*2 genes were present in 5 (35.7%) and 4 isolates respectively (Table 3.2.4).

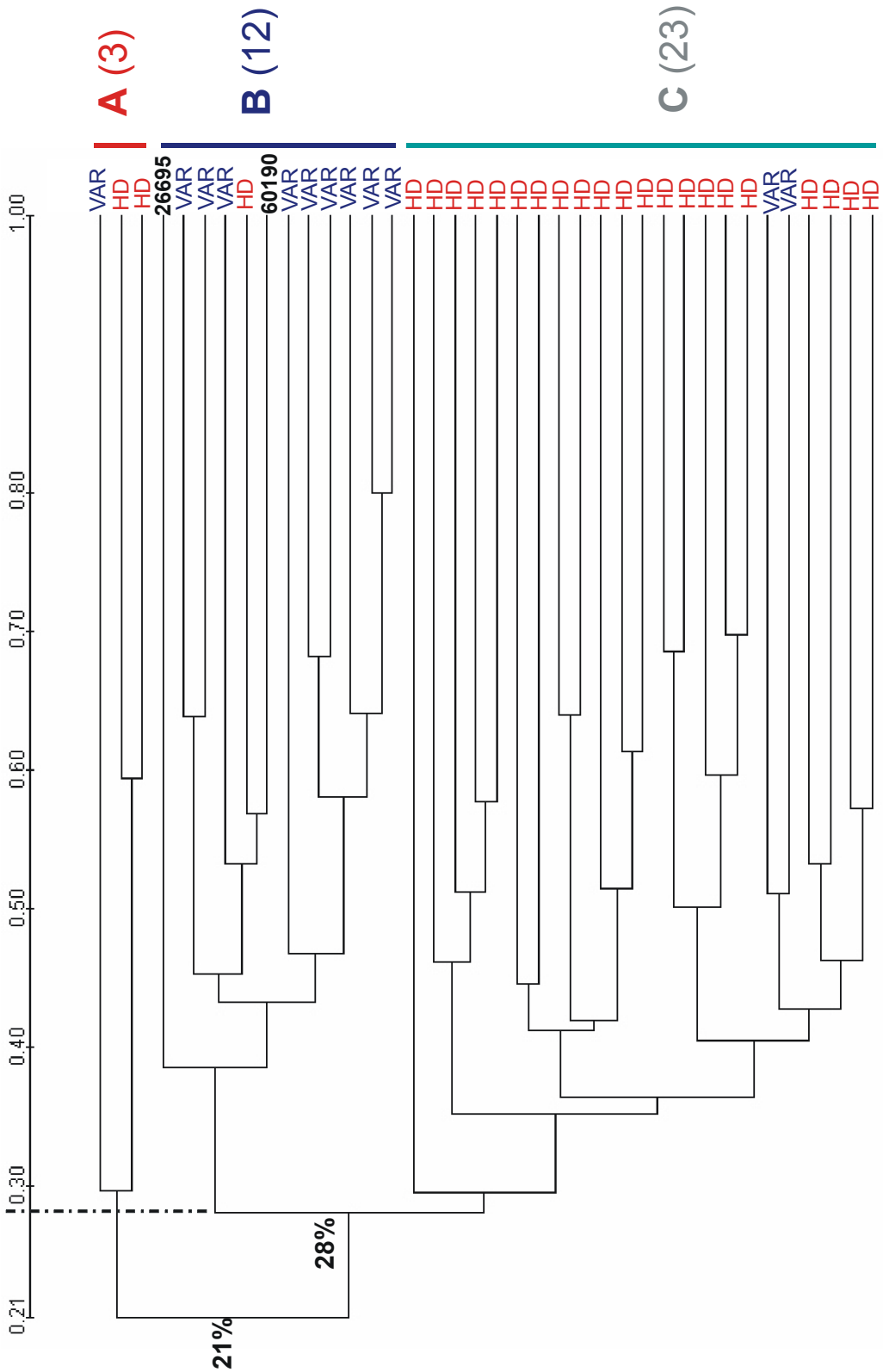


Fig. 3.2.7 Dendrogram constructed on the basis of AFLP types of entire genome. All the clinical isolates (36) and two reference strains could be placed in 3 major groups.

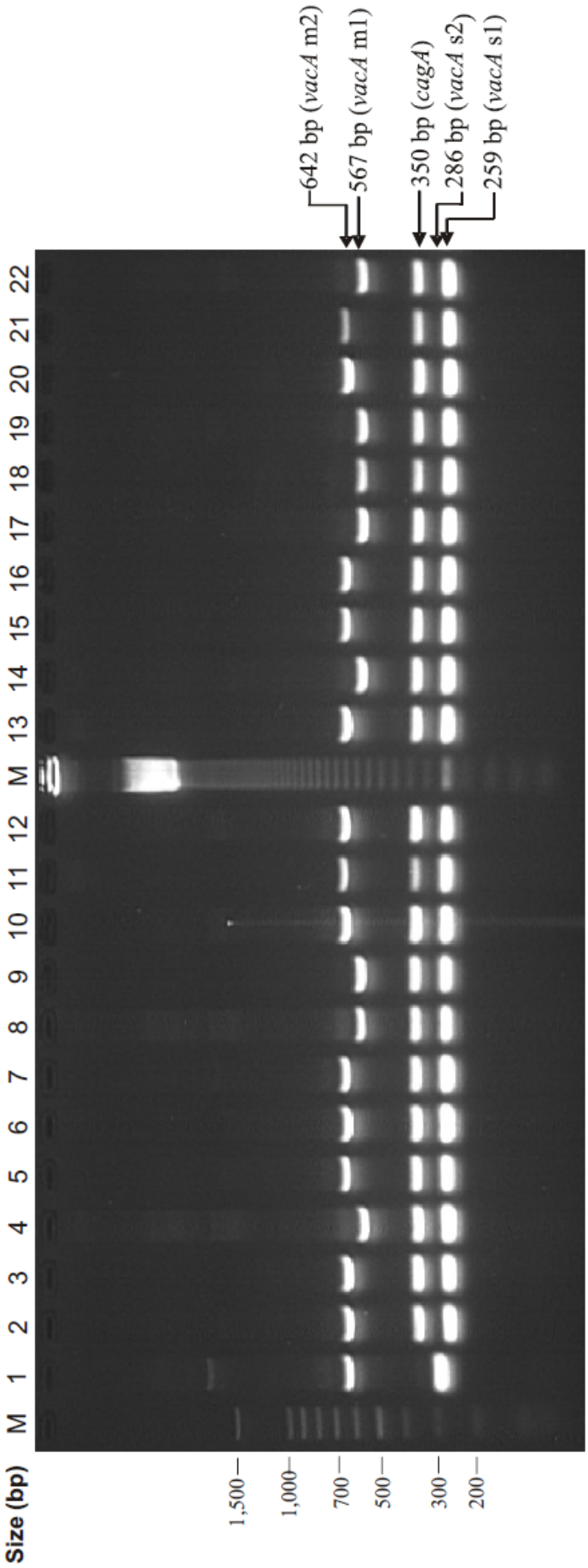


Fig. 3.2.8 Pathogenicity genes status by multiplex PCR for detection of *vacA* s1/s2, *vacA* m1/m2 and *cagA* gene of Hyderabad isolates. Lane M-100bp Ladder (left), -50bp (middle), 1-HD1, 2-HD2, 3-HD3, 4-HD4, 5-HD5, 6-HD6, 7-HD7, 8-HD8, 9-HD9, 10-HD10, 11-HD11, 12-HD12, 13-HD13, 14-HD14, 15-HD15, 16-HD16, 17-HD17, 18-HD18, 19-HD19, 20-HD20, 21-HD21, and 22-HD22.

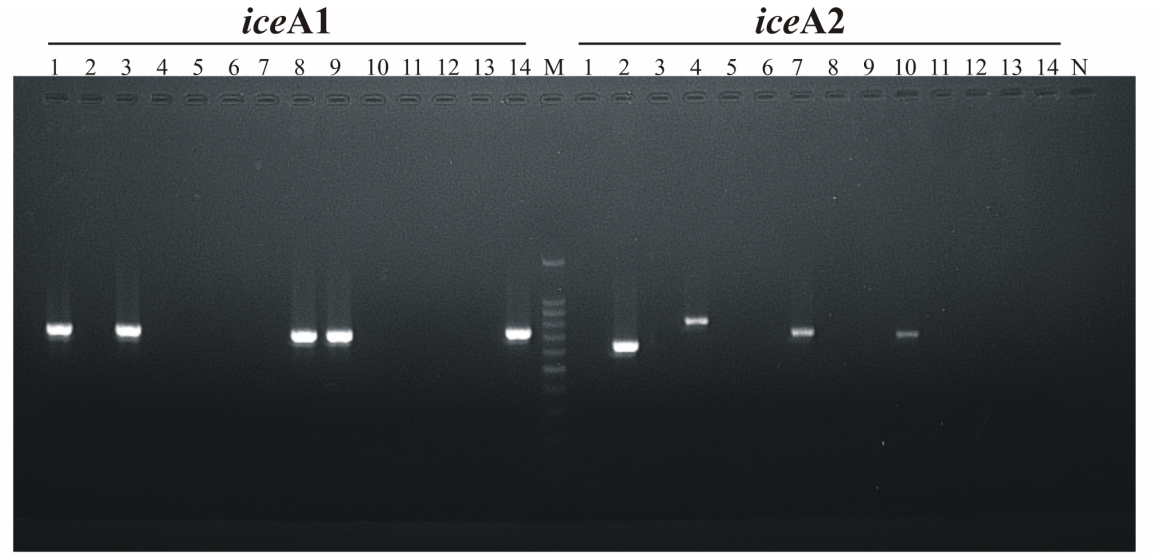


Fig. 3.2.9 Typical representation of pathogenicity genes status of Varanasi isolates. Amplification of *iceA*1 and *iceA*2 of Varanasi isolates. Lanes; M-100bp Ladder (middle), 1-VAR1, 2-VAR2, 3-VAR3, 4-VAR4, 5-VAR5, 6-VAR6, 7-VAR7, 8-VAR8, 9- VAR9, 10-VAR10, 11-VAR11, 12-VAR12,13-VAR13, 14-VAR14, and N - negative control (without template).

Table 3.2.3 Status of pathogenicity genes (*vacA* s1/s2, *vacA* m1/m2, *cagA*, *iceA*1 and *iceA*2) in various isolates of Hyderabad

S. No.	Sample ID	*vacA* signal		*vacA* middle		*cagA*	*iceA*1	*iceA*2
		s1	s2	m1	m2			
1	HD1	-	+	-	+	-	+	-
2	HD2	+	-	-	+	+	+	-
3	HD3	+	-	-	+	+	+	-
4	HD4	+	-	+	-	+	+	-
5	HD5	+	-	-	+	+	-	+
6	HD6	+	-	-	+	+	+	-
7	HD7	+	-	-	+	+	+	-
8	HD8	+	-	+	-	+	-	+
9	HD9	+	-	+	-	+	-	+
10	HD10	+	-	-	+	+	+	-
11	HD11	+	-	-	+	+	-	+
12	HD12	+	-	-	+	+	-	+
13	HD13	+	-	-	+	+	+	-
14	HD14	+	-	+	-	+	+	-
15	HD15	+	-	-	+	+	+	-
16	HD16	+	-	-	+	+	+	-
17	HD17	+	-	+	-	+	+	-
18	HD18	+	-	+	-	+	+	-
19	HD19	+	-	+	-	+	+	-
20	HD20	+	-	-	+	+	+	-
21	HD21	+	-	-	+	+	+	-
22	HD22	+	-	+	-	+	+	-
Total (%)		**21 (95.5)**	**1 (4.5)**	**8 (36.4)**	**14 (63.6)**	**21 (95.5)**	**17 (77.3)**	**5 (22.7)**

Table 3.2.4 Status of pathogenicity genes (*vacA* s1/s2, *vacA* m1/m2, *cagA*, *iceA*1 and *iceA*2) in different isolates of Varanasi

S. No.	Sample ID	*vacA* signal		*vacA* middle		*cagA*	*iceA*1	*iceA*2
		s1	s2	m1	m2			
1	VAR1	+	-	-	+	+	+	-
2	VAR2	-	+	-	+	-	-	+
3	VAR3	+	-	+	-	+	+	-
4	VAR4	+	-	+	-	-	-	+
5	VAR5	+	-	-	+	+	-	-
6	VAR6	+	-	+	-	-	-	-
7	VAR7	+	-	+	-	+	-	+
8	VAR8	+	-	+	-	+	+	-
9	VAR9	+	-	+	-	+	+	-
10	VAR10	+	-	+	-	+	-	+
11	VAR11	+	-	+	-	+	-	-
12	VAR12	+	-	-	+	+	-	-
13	VAR13	+	-	-	+	+	+	-
14	VAR14	+	-	+	-	+	-	-
Total (%)		**13 (92.9)**	**1 (7.1)**	**9 (64.3)**	**5 (35.7)**	**11 (78.6)**	**5 (35.7)**	**4 (28.6)**

3.8 Phylogenetic analysis of isolates based on 16S rDNA sequences

Phylogenetic analysis based on 16S rDNA sequences clearly showed that isolates of Hyderabad and Varanasi get clustered separately and show significant diversity among each other (Fig 3.2.10 and Table 3.2.5). It is also evident from the results that the level of diversity was more prominent among the isolates of Varanasi (North India) in comparison to the isolates of Hyderabad. Additionally if comparison is made with the isolates of other countries (Fig 3.2.11), Varanasi isolates showed homology with the isolates of Taiwan except VAR 10 and VAR13 which shared homology with a isolate of Brazil. On the other hand majority of the isolates of Hyderabad matched with the isolates of Brazil excluding isolate HD3 which showed homology with one isolate of Taiwan. Altogether results of

phylogenetic analysis revealed that isolates of North and South India show distinct diversity and Indian isolates show close homology either with the isolates of Brazil or Taiwan.

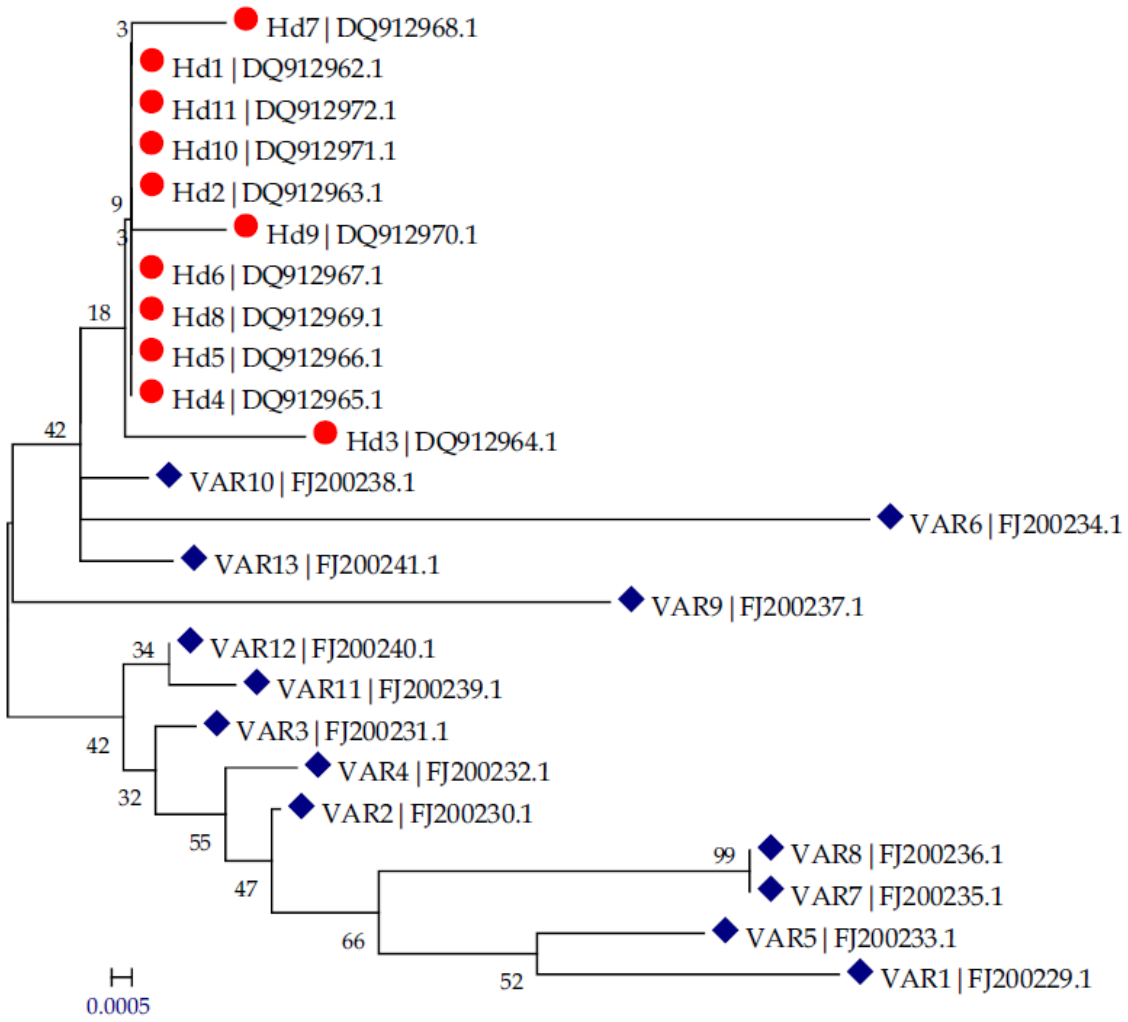


Fig. 3.2.10 Evolutionary relationships based on partial 16S rDNA sequences of 24 (Varanasi 13 and Hyderabad 11) Indian taxa The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 20% bootstrap replicates are collapsed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. There were a total of 456 positions in the final dataset. Phylogenetic analyses were made in MEGA4.

Table 3.2.5 Estimates of evolutionary divergence between Varanasi and Hyderabad sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. VAR8|India																							
2. VAR7|India	0.000																						
3. VAR5|India	0.023	0.023																					
4. VAR1|India	0.014	0.014	0.012																				
5. VAR4|India	0.014	0.014	0.014	0.019																			
6. VAR2|India	0.012	0.012	0.009	0.016	0.002																		
7. VAR3|India	0.016	0.016	0.012	0.016	0.007	0.005																	
8. VAR10|India	0.021	0.021	0.019	0.023	0.007	0.009	0.007																
9. VAR9|India	0.033	0.033	0.037	0.040	0.021	0.019	0.021	0.023															
10. VAR6|India	0.035	0.035	0.035	0.035	0.031	0.028	0.023	0.021	0.038														
11. VAR12|India	0.016	0.016	0.014	0.019	0.005	0.005	0.002	0.005	0.014	0.024													
12. VAR11|India	0.016	0.016	0.014	0.021	0.007	0.005	0.005	0.009	0.016	0.026	0.000												
13. VAR13|India	0.023	0.023	0.023	0.026	0.012	0.012	0.009	0.007	0.018	0.023	0.002	0.007											
14. HD3|India	0.023	0.023	0.023	0.028	0.014	0.014	0.012	0.007	0.021	0.026	0.009	0.014	0.007										
15. HD11|India	0.021	0.021	0.019	0.023	0.009	0.009	0.007	0.002	0.016	0.021	0.005	0.009	0.002	0.005									
16. HD7|India	0.023	0.023	0.021	0.026	0.012	0.012	0.009	0.005	0.019	0.023	0.007	0.012	0.005	0.007	0.002								
17. HD4|India	0.021	0.021	0.019	0.023	0.009	0.009	0.007	0.002	0.016	0.021	0.005	0.009	0.002	0.005	0.000	0.002							
18. HD5|India	0.021	0.021	0.019	0.023	0.009	0.009	0.007	0.002	0.016	0.021	0.005	0.009	0.002	0.005	0.000	0.002	0.000						
19. HD1|India	0.021	0.021	0.019	0.023	0.009	0.009	0.007	0.002	0.016	0.021	0.005	0.009	0.002	0.005	0.000	0.002	0.000	0.000					
20. HD6|India	0.021	0.021	0.019	0.023	0.009	0.009	0.007	0.002	0.016	0.021	0.005	0.009	0.002	0.005	0.000	0.002	0.000	0.000	0.000				
21. HD2|India	0.021	0.021	0.019	0.023	0.009	0.009	0.007	0.002	0.016	0.021	0.005	0.009	0.002	0.005	0.000	0.002	0.000	0.000	0.000	0.000			
22. HD8|India	0.021	0.021	0.019	0.023	0.009	0.009	0.007	0.002	0.016	0.021	0.005	0.009	0.002	0.005	0.000	0.002	0.000	0.000	0.000	0.000	0.000		
23. HD9|India	0.023	0.023	0.021	0.026	0.012	0.012	0.009	0.005	0.019	0.023	0.007	0.012	0.005	0.007	0.002	0.005	0.002	0.002	0.002	0.002	0.002	0.002	
24. HD10|India	0.021	0.021	0.019	0.023	0.009	0.009	0.007	0.002	0.016	0.021	0.005	0.009	0.002	0.005	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.002

The number of base substitutions per site from analysis between sequences is shown. All results are based on the pairwise analysis of 24 sequences. Analyses were conducted using the Kimura 2-parameter method in MEGA4. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 456 positions in the final dataset.

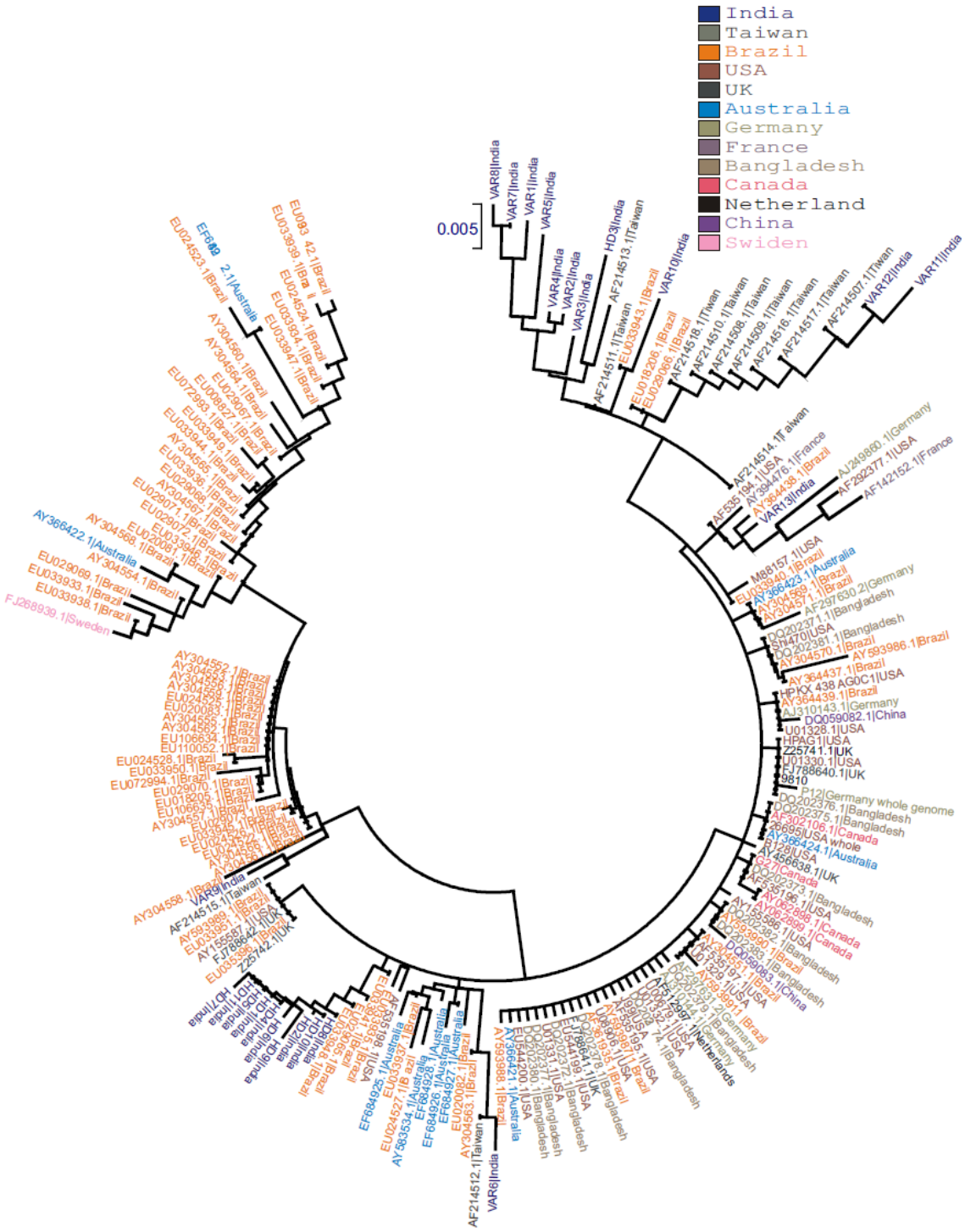


Fig. 3.2.11 Evolutionary relationships of 184 taxa. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 512 positions in the final dataset. Phylogenetic analyses were made in MEGA4.

4 Discussion

Remarkable genetic variations have been reported between *H. pylori* strains isolated from individuals, yet the origins and determinants of this diversity are not completely understood and may not be observed by all typing methods. It is possible that there is a relation between the diverse gastric pathologies seen in humans and diversity of *H. pylori*, since this organism is closely associated with the diseases. However, it is essential to have good typing procedures for *H. pylori* before a clear understanding of these diseases and any definitive relationship to given *H. pylori* strains can be established. These should be essential in allowing us to interpret phylogenetics and the population genetics of *H. pylori*, as well as reservoirs, routes, and modes of transmission.

There are several approaches which are frequently used in phylogenetic studies specifically based on the rDNA sequences, electrophoretic pattern obtained after restriction digestion of the 16S rDNA amplicon. Among all ARDRA is one of the simple and convenient methods for deriving phylogenetic relatedness. This technique, popularly known as Amplified Ribosomal DNA Restriction Analysis (ARDRA) has been successfully used to analyze phylogenetic relatedness in the bacterial communities (Heyndrickz *et al*., 1996). Taking into account the above approach in this study, 16S rDNA was amplified in selected 36 isolates with a pair of primer designed in the conserved region of the gene (Weisburg *et al*., 1991) and digested with two tetra-cutting restriction endonucleases in order to reveal the restriction fragment length polymorphism of 16S rDNA. On the basis of shared DNA fragment analysis, the isolates were found to be phylogenetically different among each other. Similar to our study, PCR-RFLP analysis of small subunit of 16S rDNA has been used for grouping large number of bacteria (Lafay and Burdon, 2001).

No doubt ARDRA did show genetic diversity among all the 36 isolates, however rDNA- based fingerprinting is not very useful tool for discrimination of bacteria above the strain level, as it lays too much emphasis on a single locus for deciphering phylogenetic affinities. Therefore multilocus analysis like ERIC-PCR and AFLP are preferred methods for rapid analysis of molecular polymorphism among bacterial isolates. As such analysis of bacterial isolates for molecular diversity or taxonomic assignment may not be judged by any one PCR-based assay and therefore it is always useful to apply a number of molecular tests. In general the distribution of repetitive sequences (ERIC) has been employed in elucidating the genomic diversity in a number of bacteria

(Selenska-Pobell *et al*., 1995) and amplification of inter-REP elements has been frequently used to detect similarities in a given group of bacteria. Since repetitive sequences such as ERIC- elements are ubiquitous in bacteria (de Bruijn, 1992), it is presumed that they may facilitate rapid molecular characterization. In this study, the bacterial isolates were analyzed by ERIC-PCR where the presence of 1-12 bands was noted. Reproducibility of the ERIC profile was evident from the fact that identical banding pattern was obtained when replicate samples were run together. ERIC-PCR showed the presence of large number of bands suggesting that there is indeed presence of repetitive sequences in the genome of all the 36 isolates. Several other workers have also applied ERIC-PCR for studying the bacterial diversity including *H. pylori* (Hussain *et al.,* 2004). Our results are in agreement with the above report.

AFLP analysis is a technique in which adapter molecules are ligated to restriction enzyme digested fragments and the ligated product is used as target sites for primers in the PCR amplification process (Gibson, *et al*., 1998). It can be applied for the fingerprinting of a wide variety of microbial species. Accordingly we adopted single-restriction-enzyme approach of Gibson *et al*., (1998) for typing various strains of *H. pylori*. Contrary to PCR-RFLP methods that examine restriction site changes in a single gene, AFLP analysis provides a means of examining DNA segments distributed over the entire genome of an organism. In the present study all the *H. pylori* isolates from Varanasi and Hyderabad were fingerprinted by using *Hind*III digestion of DNA, the results showed a high level of typeability with this enzyme.

Though AFLP analysis is not as rapid as some other PCR-based assays such as ERIC-PCR and RAPD analysis, but when compared to ribotyping performance, it is less labor-intensive and, because the profiles contain more bands, the results are more informative. Additionally the reproducibility of the AFLP profiles is always at par excellence. This was evident from our results wherein identical banding pattern was obtained when same samples were analysed several times. In our study whole genome was digested with *Hind*III followed by ligation with adapter DNA and finally amplification was done by using HI-A and HI-C primers for the analysis of AFLP. Amplification with HI-A showed 5-12 bands whereas HI-C showed 2-8 bands. Interestingly all the isolates showed distinct banding pattern. Since HI-A gave more bands than HI-C; its banding profile was used in the construction of phylogenetic tree.

The dendrogram based on the AFLP pattern was constructed using UPGMA to reveal genetic relationship among 36 isolates together with the standard ATCC reference strains (60190 and 26695). Similarity coefficient revealed that all the 38 strains could be placed into 3 major groups, namely A, B and C. Our results clearly demonstrate genomic diversity in geographically distinct isolates. It is evident from the data that group A included isolates from Varanasi as well as Hyderabad and these isolates showed least diversity i.e., 21% similarity with other group. Group B included only Varanasi isolates which included reference strains. Interestingly group C had maximum 20 (86.9%) isolates of Hyderabad and only 3 (13.1%) isolates from Varanasi. Similar to our findings Owen *et. al.* (2001) have also reported a high degree of genomic variation among isolates of *H. pylori* from different individuals. It is also interesting to note that we did not get a single isolate which showed 100% similarity with other isolate. Our results clearly demonstrate that interstrain genomic heterogeneity is a characteristic feature of *H. pylori.*

In general for long-term successful colonization in the human stomach, a set of bacterial virulence determinants would develop for initial adhesion, maintenance and altering of gastric tissue. Taking into consideration all the above factors, VacA, CagA and BabA have been shown to be related to more severe clinical outcomes (Kusters *et al*., 2006). As mentioned in previous section, notable differences in the distribution of *cagA* gene in *H. pylori* strains have been reported from different parts of the world (Matteo *et al*., 2007; Mukhopadhyay *et al*., 2000). It has been demonstrated that *cagA* gene is present in 50% to 70% of *H. pylori* strains in Western populations, wherase it is present in >90% of strains from the Eastern populations (Bolk *et al*., 2007). In the present study *cagA* gene was detected in 95.5% of isolates from Hyderabad and 78.6% of Varanasi. Further analysis revealed that *vacA* s1/m1 genotype was present in 36.4% (8) isolates of Hyderabad and 64.3% (9) in Varanasi isolates. *vacA* s1/m2 genotype was noted in 13 (59%) isolates of Hyderabad whereas 4 (28.6%) in Varanasi isolates. Interestingly *vacA* s2/m2 genotype was present in only one isolate of the above two regions. Furthermore, all the *cagA* positive isolates harboured either s1/m1 or s1/m2 combination. Additionally, out of 22 isolates of Hyderabad, 17 (77.3%) contained *iceA*1 and 5 (22.7%) *iceA*2. On the other hand, among 14 isolates of Varanasi 5 (35.7%) had *iceA*1 and 4 (28.6%) *iceA*2. It has been reported that the expression of *iceA* is upregulated when contact between *H. pylori* and human epithelial cells is established (van Doorn *et al*., 1998a). Role of *iceA*1 gene in the development of peptic ulcer disease has also been reported but several

workers have failed to confirm this correlation. There is a report suggesting that *iceA*1 positive isolates produced enhanced level of proinflammatory factor IL-8 than *iceA*1 negative isolates (Blaser *et al*., 1995). In the present study we have observed that *iceA*1 is the most frequent genotype among various *H. pylori* isolates. Our finding is in agreement with other reports where *iceA*1 allele was found more frequently than *iceA*2 in Chinese, Japanese, Korean and Dutch subjects (van Doorn *et al*., 1998a). Recently Chomvarin *et al*., (2008) have reported *iceA*1 genotype in majority of *H. pylori* strains from Thailand. On the contrary there is a report which showed that *iceA2* is the predominant subtype in the USA and Columbia (Yamaoka *et al*., 1999).

One of our main goals was to reveal genetic diversity, if any, between *H. pylori* strains isolated from patients of South and North India and to test if the gene pool of *H. pylori* belonging to this part of the world does show homology with strains of other part of the world. The analysis was made solely on the basis 16S rDNA gene sequence which is thought to be conserved. The selection of isolates from the patients of North and South region was mainly due to distinct differences in living styles, food habits and climatic conditions prevailing in these two places namely, Varanasi and Hyderabad. Altogether we analysed sequences of 24 isolates and the results showed that Varanasi isolates are much more diverse than the isolates of Hyderabad. Notably most of the Hyderabad isolates showed 100% homology among themselves whereas majority of the Varanasi isolates showed distinct differences among themselves. One of the possible reasons for close homology among Hyderabad isolates might be due to close similarity in food habits and living standard of the people as well as the prevailing climatic conditions. Majority of the people in South rely mostly on common spicy foods, weather is mostly warm and there are not drastic fluctuations in the climatic conditions. Most probably *H. pylori* senses common stresses and adapts accordingly. On the contrary, Varanasi (North) is a Holy city, people come from different parts of the world and there is mixing of culture. Dietary habits differ from person to person. Reasonably there are too much variations in climatic conditions. All these above factors might be responsible for causing diversity among *H. pylori* populations.

Comparison of Indian isolates with the isolates of other parts of the world suggests that most of the Indian isolates reported in this study get clustered separately and show close homology either with the isolates of Taiwan or Brazil. This analysis was made

from the sequences available in the data base of GeneBank, EMBL and DDBJ. It is also interesting to note that almost all the isolates of Varanasi showed homology with Taiwan whereas Hyderabad isolates shared homology with Brazil. Such differences are expected since marked differences in the isolates of Varanasi and Hyderabad were noted. Due to unavailability of sequences of 16S rDNA of Indian *H.pylori* isolates in database we could no compare our data with other isolates. To our knowledge this is the first report where an attempt has been made to construct a phylogenetic tree based on conserved gene (16S rDNA) with a view to show homology of Indian isolates at global level. Ahmed *et al*. (2003) constructed phylogenetic tree on the basis of house keeping and other genes and showed homology of Indian isolates with Europe. The differences from our report could be due to selection of different genes for tracing phylogeny. Additionally we could not get large number of sequences (part of the sequence that shows homology with our sequence) from Europe due to unavailabily in data base.

There is a general feeling that an evolving theme in genetic analysis of *H. pylori* is that this species is characterized by immense genetic diversity. Many factors may contribute to the observed high level of allelic variation (Atherton *et al*., 1995). Primarily, *H. pylori* may be an ancient bacterial species with a long evolutionary history. In addition, there is undoubtedly a very large *H. pylori* population, potentially consisting of a vast array of lineages, all evolving in relatively isolated niches within the stomachs of individual humans (Akopyanz *et al*., 1992). A high potential of mutational frequency could contribute to the observed diversity, but the rates at which streptomycin and rifampicin resistances arise in *H. pylori in vitro* are not strikingly higher than for other bacteria (Jiang *et al*., 1996). The molecular mechanisms by which variability in chromosomal gene order arises in this organism are not well understood, but multiple copies of two related insertion sequence elements (IS605 and IS606) and numerous other repeat sequences may play a major role in this phenomenon.

Horizontal DNA transfer occurrences and homologous recombination between chromosomal segments of different *H. pylori* strains are probably important factors contributing to genetic diversity. A recombinational population structure is also consistent with reports of mosaic genes in *H. pylori* (Atherton *et al*., 1995) and has been suggested based on sequence analysis of several genes.

A number of bacterial species with recombinational population structures, including *N. gonorrhoeae, Neisseria meningitides, and Streptococus pneumoniae,* are naturally transformable. *H. pylori* is also known to be a naturally transformable pathogen (Wang *et al*., 1993), and exchange of chromosomal antibiotic resistance markers between *H. pylori* strains has been demonstrated *in vitro* (Kupiers *et al*., 2006). Preliminary studies suggest that DNA exchange between *H. pylori* strains may occur via both natural transformation and bacterial conjugation (Kupiers *et al*., 2006). Simultaneous infection of individual humans with multiple *H. pylori* isolates is known to occur, and thus there may be ample opportunities for intraspecies genetic exchange *in vivo*.

In conclusion our results clearly show that genetic diversity among *H. pylori* isolates is widely prevalent among various strains isolated from a particular or different region. We have demonstrated that isolates of North and South India show distinct diversity. Furthermore results of the present study clearly demonstrate that AFLP may be conveniently employed in genotyping of various *H. pylori* isolates. More interestingly phylogenetic analysis suggests that Indian isolates show close homology either with the isolates of Taiwan and /or Brazil.

Section3

Genetic Diversity of Pathogenicity gene

cag PAI Diversity among North and South Indian Isolates

1 Introduction

The *cagA* gene is located downstream portion of the *cag* pathogenicity island (*cag* PAI). The *cag* PAI contains genes that encode a type IV secretion system and immunodominant antigen, CagA, which is translocated into gastric epithelial cells (Censini *et al*., 1996). The presence of *cagA* gene is thought to be a marker for the presence of *cag* PAI. In general infection with *cag* PAI positive strains of *H. pylori* significantly increases the risk of developing severe gastric mucosal inflammation, peptic ulceration and gastric cancer in comparison to *cag* PAI negative strains (Graham and Yamaoka, 1998; Naito *et al*., 2006; Segal *et al*., 1999).

Due to large degree of genomic and allelic diversity, there are reports of significant geographical differences among strains of *H. pylori*. It has been observed that only one half to two thirds of Western isolates carry the *cag* PAI whereas almost all the East Asian strains carry this island (Nomura *et al*., 1991; Parsonnet *et al*., 1991). Furthermore, there are reports that the *cag* PAI is highly conserved in Japanese isolates, least conserved in European and African isolates, and very poorly conserved in Indian isolates (Nomura *et al*., 1991). Interestingly strains of *H. pylori* such as, 26695 and J99, which show similarity in at least two major disease-associated loci (both are *cag* PAI positive and contain type s1 *vacA* alleles) also show distinct differences in genetic profile (Terry *et al*., 2005). This is further supported from the data of complete genome sequence which revealed that strain 26695 had 110 open reading frames (ORFs) which were absent in J99 and strain J99 contained 52 genes which were absent in strain 26695 (Terry *et al*., 2005).

It is presumed that at some point during evolution, IS*605*, a mobile sequence encoding two transposases, entered the *H. pylori* genome and in some strains interrupted, multiplied or caused partial deletion of the PAI. (Fischer *et al*., 2001). Screening of *cag* PAI genes namely, *cagA*, *cagG*, *cagE, cagM* and *cagT* has been frequently tested so as to reveal the presence of intact *cag* PAI (Fig.3.3.1) (Terry *et al*., 2005). It has been reported that *cagG* gene might be a better marker for confirming the presence of intact *cag* PAI

than the *cagE* gene especially in French isolates (Ping *et al*., 2002). However deletion of *cagE*, *cagT*, *cagA*, *cagG* and *cagM* genes has been reported in several cases of chronic gastritis, gastric ulcer and gastric cancer cases, indicating that the pathogenicity of *H. pylori* may not be determined by *cag* PAI genes alone (Covacci *et al*., 1999). It has also been reported that deletion frequencies of *cagA, cagE and cagT* genes were highest in benign cases where the left end of the *cag* PAI was frequently rearranged in isolates from severe cases. Several workers also found lack of correlation in different *cag* PAI rearrangements with clinical manifestation. All these reports clearly indicate that any change in *cag* PAI namely deletion, rearrangement etc is bound to affect the clinical status of the subjects (Covacci *et al*., 1999; Terry *et al*., 2005).

Available data show that the diversity of *cagA* gene in general and association of the *cag* PAI, either intact or partially deleted in particular with the appearance/manifestation of disease is poorly understood in patients of different locations/regions. For better understanding, there is a need to gather data on the abundance of intact versus rearranged *cag* PAI in strains inhabiting geographically and culturally distinct patient populations. Furthermore amplification of whole fragment of *cagA* would be desirable to reveal the diversity and clinical outcome. In the present study, we have made an attempt to investigate *cagA* diversity together with the analysis of *cag* PAI integrity in patients from South and North India employing PCR techniques, the emphasis being on the amplification of whole segment of *cagA* followed by RFLP analysis. To our knowledge, this approach has not been employed so far in understanding the diversity of *cagA* and /or in revealing the status of *cag* PAI in patients of distantly located regions.

2 Materials and methods

2.1 Patients and bacterial strains

Details of patients and reference isolates are described in Section 2 (2.1).

2.2 Isolation and culture of *H. pylori*

Methods for the isolation and growth of *H. pylori* were similar to those described in Section 1 (2.4.4).

2.3 DNA extraction and amplification of *cagA, cagE* and *cagT* genes

Genomic DNA was extracted by DNeasy Tissue Kit (Qiagen, GmbH, Hilden, Germany) following the instructions of manufacturer. *cagA, cagE and cagT* genes of laboratory- grown pure cultures were amplified using standard set of primers (Fig. 3.3.1 and Table 3.3.1). Amplification was performed in a PTC-100 Thermal Cycler (MJ Research). The PCR reaction mix included 0.75 U of *Taq* DNA polymerase (Bangalore Genei), 1 X PCR assay buffer with 1.5 mM $MgCl_2$, 10 pmole of the forward and reverse primers (Integrated DNA Technologies, Inc., USA), 200 µM each of the deoxynucleotide triphosphates (dNTPs) and 50 ng of template DNA in a total volume of 25 µl. Thermal cycles for the amplification were set at; initial denaturation for 5 min at 94° C, 35 cycles of 30 s at 94°C, 30 s at 52°C and 1 min at 72°C followed by final extension of 5 min at 72° C. The amplified products were electrophoresed on a 1.2% agarose gel in TAE buffer (40 mM Tris-acetate –1mM EDTA, pH 8.0) containing ethidium bromide (0.5 µg/ml) and then visualized in a gel documentation unit (Bio-Rad Laboratories). Genomic DNA of *H. pylori* 60190 (have s1/m1 genotype and is *cagA* positive) was used as reference isolate.

Table 3.3.1 Primers used for the amplification of *cag* PAI

Primer	Primer sequence (5'-3')	Product Size(bp)	References
cag*A*5	GGCAATGGTGGTCCTGGAGCTAGGC	325	Mukhopadhyay *et al*., 2000
cag *A*2	GGAAATCTTTAATCTCAGTTCGG		
cag*A*-F40481	AGGATTTCATCAAGGTAACGCAAGC	1253	Mukhopadhyay *et al*., 2000
cag*A*-R41660	TAAGATTTTTGGAAACCACTTTTGTAT		
cag*E*-F1	GCGATTGTTATTGTGCTTGTAG	329	Ikenoue *et al*., 2001
cag*E*-R1	GAAGTGGTTAAAAAATCAATGCCCC		
cag*T*-F1	CCATGTTTATACGCCTGTGT	301	Ikenoue *et al*., 2001
cag*T*-R1	CATCACCACACCCTTTTGAT		

F, Forward; R, Reverse; A5 (F); A2 (R).

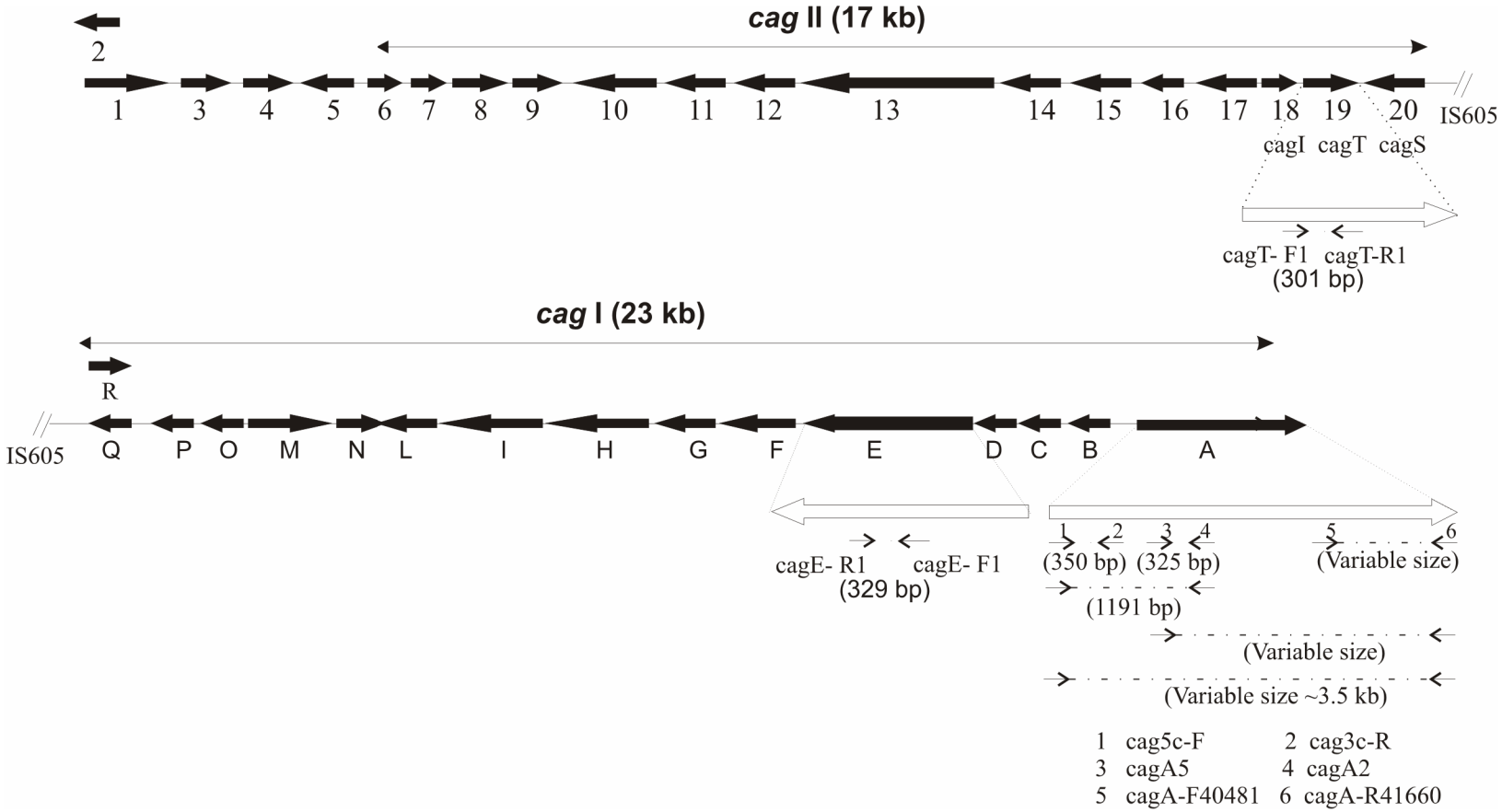


Fig. 3.3.1 Structure of *cag* pathogenicity island showing location of various genes (dark arrow). Location of *cagA*, *cagE* and *cagT* genes has been denoted by dotted box and respective open arrow. Three set of primers were used to detect consensus and variable region of *cagA* gene, first set was *cag*5c-F and *cag*3c-R, second was *cagA*5 and *cagA*2 and, the third set was *cagA*-F40481 and *cagA*-R41660 (region of each primer combination denoted by arrow). Different primer combinations were also used to amplify *cagA* gene of varying sizes. The sequence of each primer is given in Table 3.3.1.

2.4 Amplification of the large fragment and 3' variable region of *cagA*

Amplification of large fragment and 3' variable region was performed using primer set *cag*5c-F and *cagA*-R41660 and *cagA*-F40481 and *cagA*-R41660 respectively. The PCR reaction mix included 1.5 U of *Taq* DNA polymerase (Bangalore Genei), 1 X PCR assay buffer with 1.5 mM $MgCl_2$, 10 pmole each of the forward and reverse primers (Integrated DNA Technologies, Inc.), 200 µM each of the dNTPs and 50 ng of template DNA in a total volume of 50 µl. Thermal cycle for the amplification was set at; initial denaturation for 5 min at 94° C, 35 cycles of 1 min at 94° C, 1 min at 52° C and 3 min at 72° C followed by final extension of 10 min at 72° C. The amplified products were analyzed as mentioned above.

2.5 RFLP of large fragment of *cagA*

Forty microlitres of the amplified PCR product were removed and ethanol precipitated and resuspended in 20 µl of deionised water. Out of 20 µl purified DNA, 10 µl was digested with 10 U of the restriction enzymes *Hind*III and *Alu*I for 16 h at 37° C as per the instructions of manufacturer (Promega). The digested products were run together with a 100-bp DNA ladder in a 3% agarose gel at 100 V in TAE buffer for 4–5 h. Cluster analysis of RFLP types was performed by UPGMA (unweighted pair- group method with arithmetic averages) using Quantity One® 1-D Analysis Software, version 4.4 (Bio-Rad Laboratories). Banding pattern similarities were scored using Dice coefficient.

3 Results

3.1 Detection of *H. pylori* in different patients

H. pylori was successfully isolated from the biopsy samples of all the thirty six patients belonging to Varanasi, North India (14) and Hyderabad, South India (22). Identification was made on the basis of morphological characters, biochemical tests and amplification of *H. pylori* specific 16S rDNA (Kumar *et al*., 2008). Results of upper gastrointestinal endoscopy revealed that out of 36 patients, 31 had different types of gastrointestinal diseases and 5 was normal with no evidence of gastric diseases. Among the 14 patients of North India (Varanasi), 4 had gastritis, 3 gastric cancer (GC), 2 duodenal ulcer (DU), 1 gastric ulcer (GU) and 4 was normal as evident from the

endoscopic investigation.. Similarly, among patients of Hyderabad (South India), 13 had DU, 4 GU, 2 GC, 2 gastritis and 1 was normal (showing no signs of any gastrointestinal disease).

3.2 Characteristics of *cag* PAI regions

Five pairs of primers were used to detect the presence of the *cagA*, *cagE* and *cagT* genes in various *H .pylori* isolates. Three sets were used to detect the *cagA* gene, two for 5' and one for 3' end. For elucidating the presence of *cagE* and *cagT*, one set of primer was used for each gene. *H. pylori* strain ATCC 60190, which is known to contain the entire *cag* PAI region served as positive control for each PCR assay. Typical representation of PCR amplified product of all the genes are shown in Fig. 3.3.2. Amplified product of *cagA* 5' end, *cagE* and *cagT* genes was confirmed further by sequencing of selected Varanasi isolates and the result showed 97-99% homology with the sequences available in data base (Section 2, Table 3.2.2). Analysis of data as represented in Table 3.3.2 shows the distribution pattern of *cagA*5' end, *cagA*3' end, *cagE* and *cagT* genes in various isolates of Varanasi and Hyderabad. Results showed appreciable differences in the prevalence of the above genes among the isolates of North and South India. Distribution pattern of *cagA* revealed that out of 22 strains of Hyderabad, *cagA* 5' region was present in 21 strains (95.4%), the value being 13 (DU), 3 (GU), 2 (GC), 2 (gastritis) and 1 for normal. On the other hand *cagA* 3' region was present in all the 22 strains. Among the isolates of Varanasi (14), 11 (78.57%) strains showed the presence of *cagA* 5' region where as *cagA* 3' region was detected in 13 (92.85%) strains (Table 3.3.2). The *cagE* was detected in 21 strains of Hyderabad and 13 strains of Varanasi. The *cagT* was present in 21 strains of Hyderabad and 11 in the strains of Varanasi.

Furthermore it is evident from the results that out of 22 strains isolated from the patients of Hyderabad, 2 (9%) showed partial deletion and the remaining 20 (91%) had intact *cag* PAI (Table 3.3.3). On the other hand, strains from the patients of Varanasi (14), 5 (35.7%) showed partial deletion and 9 (64.3%) contained intact *cag* PAI. However none of the strains either from Hyderabad (South) or Varanasi (North India) exhibited complete deletion of *cag* PAI.

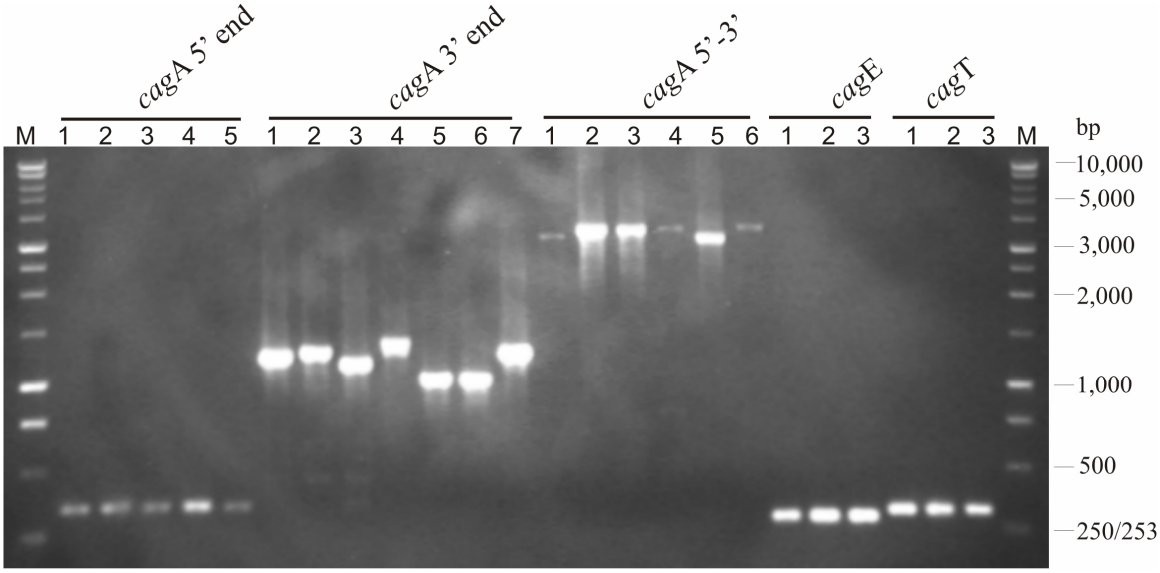


Fig. 3.3.2 Representative PCR amplification of *cagA* 5', *cagA* 3' end, *cagA* 5'-3', *cagE* and *cagT* genes by PCR. Lane M (both in left and right side) denotes 1 kb ladder (Promega). Marker size (in bp) is indicated in the right side of the gel photograph. Lane1; standard *H. pylori* strain (ATCC strain 60190), Lanes 2 to 7: amplification of desired genes as mentioned on top of gel from selected isolates of Varanasi and Hyderabad. All the remaining isolates also showed the amplification of their respective amplicons.

3.3 Analysis of the *cagA* 3' variable region

With a view to examine variations in the *cagA* variable region, PCR amplification of *cagA* 3' variable region was performed and size of the amplified product was determined. The size of PCR product of all the isolates was in the range of ca. 1042 to 1360 bp. Among the strains belonging to Varanasi region, the size was in the range of 1042 to 1267 bp whereas the range was 1167 to 1360 bp in the strains of Hyderabad. Based on the size of products, all the strains could be placed in six groups. Accordingly group I included molecular size of 1042 bp and comprised 2 (15.4%) strains of Varanasi. Group II with a molecular size of 1165 bp contained one (7.7%) strain of Varanasi and one (4.5%) from Hyderabad. Group III included the standard ATCC strain (60190) with a size of 1234 bp. Group IV having molecular size of 1267 bp included 10 (76.9%) and 19 (86.5%) strains of Varanasi and Hyderabad respectively and consisted of the largest group. Groups V and VI with a molecular size of 1285 and 1360 bp respectively included 1 (4.5%) strain each and was representative of Hyderabad strains.

Table 3.3.2. Distribution pattern of consensus 5'region and variable 3' region of *cagA*, *cagE* and *cagT* genes in various *H. pylori* isolates

Clinical Status	Region and number of *H. pylori* Isolates		*cagA* 5' end		*cagA* 3' end		*cagE*		*cagT*	
	VAR	**HYD**	**VAR**	**HYD**	**VAR**	**HYD**	**VAR**	**HYD**	**VAR**	**HYD**
DU	2	13	2 (100)	13 (100)	2 (100)	13 (100)	2 (100)	12 (92.3)	2 (100)	12 (92.3)
GU	1	4	1 (100)	3 (75)	1 (100)	4 (100)	1 (100)	4 (100)	0	4 (100)
GC	3	2	3 (100)	2 (100)	3 (100)	2 (100)	2 (66.6)	2 (100)	3 (100)	2 (100)
Gastritis	4	2	3 (75)	2 (100)	3 (75)	2 (100)	4 (100)	2 (100)	3 (75)	2 (100)
Normal	4	1	2 (50)	1 (100)	4 (100)	1 (100)	4 (100)	1 (100)	3 (75)	1 (100)
Total	**14**	**22**	**11 (78.57)**	**21 (95.4)**	**13 (92.8)**	**22 (100)**	**13 (92.8)**	**21 (95.4)**	**11 (78.57)**	**21 (95.4)**

VAR; Varanasi, HYD; Hyderabad, Figures in parenthesis denote %.

Table 3.3.3 Geographical distribution and relationship between the presence of *cag* PAI and the clinical status

Disease	Location and number of patients		Status of *cag* PAI			
			Intact PAI		Partially deleted PAI	
	VAR	HYD	VAR	HYD	VAR	HYD
DU	2	13	2 (100.0)	13 (100.0)	0	0
GU	1	4	0	3 (75.0)	1 (100.0)	1 (25.0)
GC	3	2	2 (66.6)	2 (100.0)	1 (33.3)	0
Gastritis	4	2	3 (75.0)	2 (100.0)	1 (25.0)	0
Normal	4	1	2 (50.0)	0	2 (50.0)	1 (100)
Total	**14**	**22**	**9 (64.3)**	**20 (91.0)**	**5 (35.7)**	**2 (9.0)**

VAR; Varanasi, HYD; Hyderabad, Figures in parenthesis denote %.

3.4 RFLP analysis to detect diversity in *cagA*

Analysis of *cagA* diversity among various strains of *H. pylori* was further tested by the amplification of approx. 3.5 kb *cagA* gene using forward primer for *cagA*-5' end and reverse primer for *cagA*-3' end. Amplification was successfully achieved in 21 (95.45%) strains of Hyderabad, 11 (78.57%) from Varanasi and 1 reference strain. Representation of digested products with *Hind*III and *Alu*I from 19 isolates of Hyderabad and 1 reference strain is given in Fig. 3.3.3AB. Altogether 29 distinguishable RFLP types from 32 isolates were observed (Fig. 3.3.4). It is evident from the gel photograph (Fig. 3.3.3 AB) that RFLP types were more distinct following digestion with *Alu*I in comparison to *Hind*III.

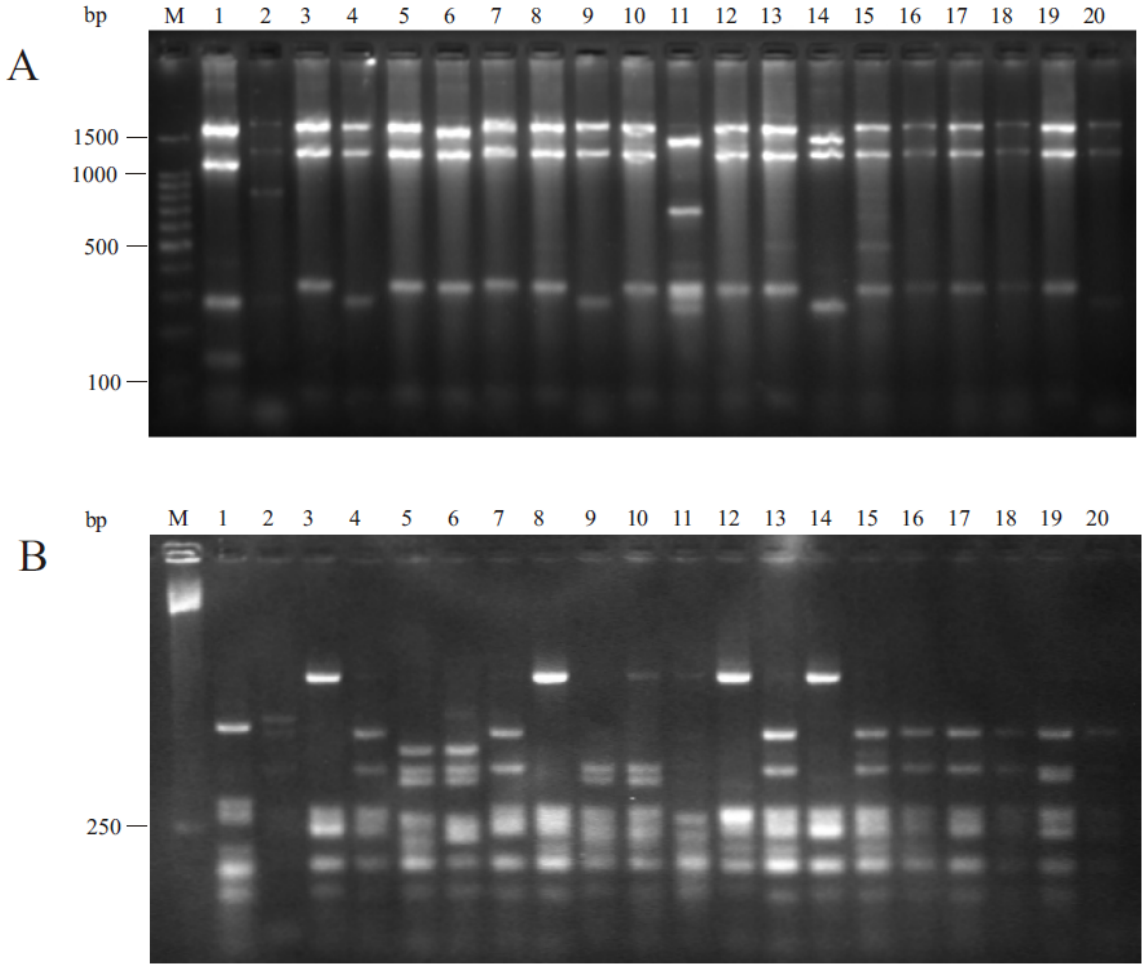


Fig. 3.3.3 AB Typical representation of RFLP pattern of 3.5 kb *cagA* gene of *H. pylori*. **A**: *Hind*III digested- lanes 2 to 20; selected isolates from Hyderabad (South India) and lane-M 100 bp (Promega). **B**: *Alu*I digested- lanes 2 to 20; selected isolates from Hyderabad (South India), and lane M-50 bp ladder (Amersham Pharmacia Biotech., Inc., Uppsala, Sweden). Lane1 of each gel represents reference *H. pylori* strain ATCC 60190.

3.5 Phylogenetic analysis of RFLP

A dendrogram based on the RFLP pattern of *cagA* was constructed using UPGMA to reveal genetic relationship among 32 clinical isolates along with standard ATCC reference strain (60190). Similarity coefficient revealed that all the 32 strains could be placed in 5 major groups, namely A, B, C, D and E (Fig. 3.3.4). Group E contained 5 strains from Varanasi and showed 54% similarity with the strains placed in groups A, B, C and D. Group D included 4 isolates, 3 from Varanasi and 1 from Hyderabad and shared 60% similarity with the strains of groups A, B and C. Group C contained 4 strains; 2 each

from Varanasi and Hyderabad and showed 64.8% similarity with the isolates of groups A and B. Group B emerged as an interesting group since it contained 18 strains , all belonging to Hyderabad and showed 66% similarity with the strains of group A. Group A consisted 2 isolates, one from Varanasi and one reference isolate. Among all the isolates, V9 and V14 from group D and H16, H17 and H18 from group B showed 100% similarity (Fig. 3.3.4).

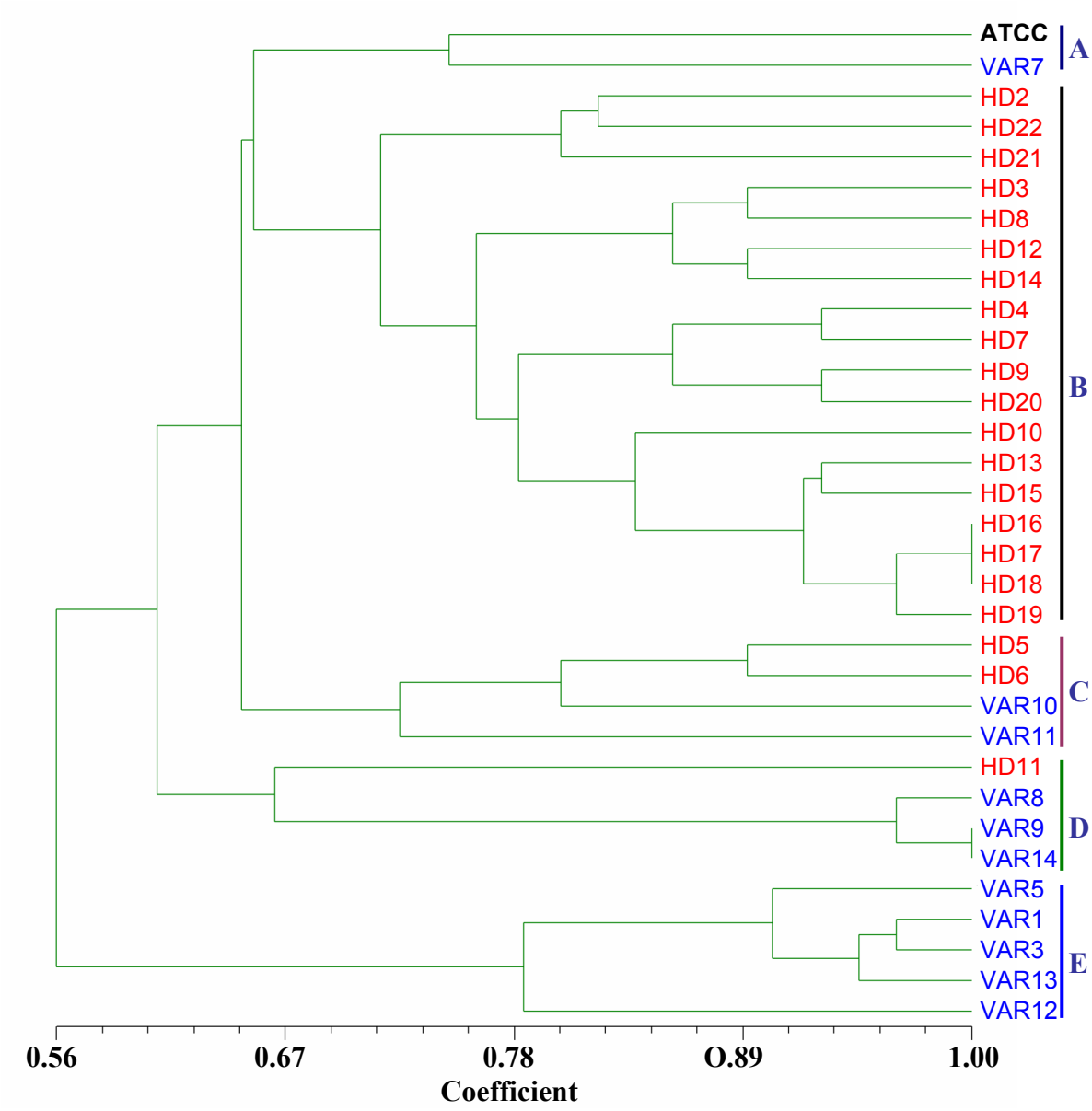


Fig. 3.3.4 Dendrogram constructed on the basis of PCR-RFLP types of *cagA* gene. All the clinical isolates (32) and one reference strain could be placed in 5 major groups. Group A had least number of isolates and group B had the maximum number of isolates.

VAR- Varanasi and HD- Hyderabad

4. Discussion

The *H. pylori* genome is genetically the most diverse among all the other bacterial species as demonstrated by the presence of non-conserved DNA fragments of *cagA* gene in the *cag* PAI and allelic variations within the *vac*A gene (Salih *et al*., 2007). Among many virulence markers present in the *H. pylori* genome, *cag* PAI is a major virulence factor and is associated with severe gastroduodenal pathology (Censini *et al*., 1996; Guillemin *et al*., 2002). Partially deleted *cag* PAI was reported in 1996 (Censini *et al*., 1996; Covacci *et al*., 1999) and since then several reports showing rearrangements in this island have appeared. The molecular mechanism of the above genetic rearrangements was explained by incorporation of an insertion element, IS605, in *cag* PAI. Recently, the composition of the *cag* PAI in clinical *H. pylori* isolates has been studied in different populations by various methods, including PCR, dot blotting and long distance PCR (Ikenoue *et al*., 2001; Jenks *et al*., 1998; Maeda *et al*., 1999). In the present study, prevalence of *cag* PAI by PCR assay in clinical isolates of *H. pylori* from patients of distantly located regions (North and South India) differing mainly in food habit, economic status and climatic conditions was studied. Results clearly demonstrated that out of 32 clinical isolates, 64.2% strains of Varanasi (North) and 90.9% from Hyderabad (South India) were carrying intact *cag* PAI. Similar to our results differences in the percentage of intact *cag* PAI in strains isolated from different parts of the world have been reported (Kauser *et al*., 2005; Mehaboob *et al*., 2005; Mukhopadhyay *et al*., 2000). Kauser *et al*. (2005) while screening the presence of *cag* PAI in isolates from the patients of eight countries found that *cag* PAI is disrupted in majority of the isolates. According to them *cag* PAI was highly conserved in Japanese isolates (57.1 %) and the least was in European and African strains. They reported that only 18.6% of the Peruvian and 12 % of the Indian isolates carried an intact *cag* PAI. In other reports, Mehaboob *et al*. (2005) found 37.4% intact *cag* PAI in Hyderabad strains, however Mukhoupdhyay *et al*. (2000) reported more than 96% in Calcutta strains of peptic ulcer and non-ulcer dyspepsia. From the findings reported herein it is evident that there are appreciable differences among strains of North (Varanasi) and South India (Hyderabad) and the percentage of intact *cag* PAI differs to some extent from the previous reports (Mehaboob *et al*., 2005; Mukhopadhyay *et al*., 2000; Kauser *et al*., 2005). Other notable difference in this finding relates to the presence of *cag* PAI and the clinical status. Herein intact *cag* PAI was detected in all the DU strains (100%) from Varanasi and the occurrence was 100% in GC,

DU and gastritis cases of Hyderabad strains. Somehow Mehaboob *et al*. (2005) reported very low (6.9%) percentage of intact *cag* PAI in DU of Hyderabad strains. Although our findings differ with those reported by Mehaboob *et al*. (2005), nevertheless are in accordance with other reports where differences in prevalence of intact *cag* PAI have been reported between different populations and within same groups (Kauser *et al*., 2005).

Studies conducted in Calcutta showed that strains having totally deleted *cag* PAI were associated with ulcer and more benign infections (Mukhopadhyay *et al*., 2000). In the West, strains lacking the *cag* PAI were recovered disproportionately from persons with benign infections (Rhead *et al*., 2007). Somehow we could not come across with a single strain showing complete deletion of *cag* PAI. It has been suggested that the presence of repeat sequences in the 3' region of the *cagA* gene may result in the production of proteins with different sizes (Covacci *et al*., 1993). This size variation raises an intriguing possibility that the biological activity of CagA can vary from one strain to the next, which may influence the pathogenicities and immunogenicity of different strains (Azuma *et al*., 2002; Higashi *et al*., 2002b). These data suggest that *cagA* variants may provide new markers for other factors involved in gastric carcinogenesis, or they may be associated with higher levels of immune response, possibly influencing the outcome of *H. pylori* infection (Yamaoka *et al*., 1998a).

Knowing that the sequence of the *cagA* gene differs from one geographic region to another, we became interested to study the variation in the size of the *cagA*3' region. Some differences in molecular size were observed between North and South Indian strains, and accordingly all the strains were grouped into 6 subtypes. However, similarity was noted in group IV which contained large number of isolates both from North and South India. Furthermore four patients, 3 from Varanasi and one from Hyderabad, that were *cagA* negative with primers for 5' region were *cagA* positive after amplification with primers for 3' variable region. Although the number of cases is too low but it appears that 3' variable region might be a better marker for the study of *cagA* typing and genetic variation. Contrary to our findings, Rota *et al*. (2001) observed that patients which were *cagA* negative after amplification with primers for the variable sequence turned out to be positive by using primers for consensus region. This ambiguity may be resolved if the study is carried out with large number of patients and selecting a variety of primers

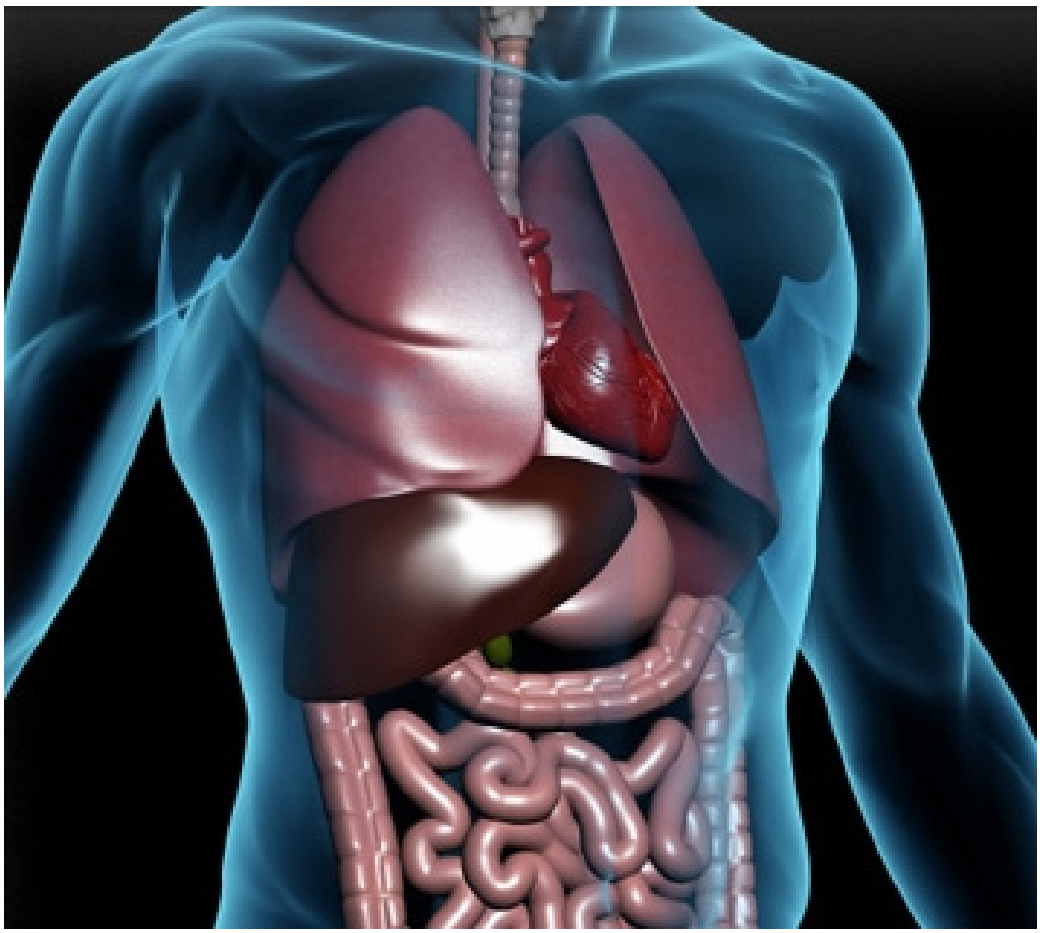
from 5' and 3' variable region. It has been reported that diversity in *H. pylori* would be enhanced if humans differ in their diet habits (gastric environments) as well as by traits in individual strains such as specific immune responses and/ or availability of receptors helpful in adhesion (Ahmed *et al*., 2003). However, the pattern of transmission in populations depends on lifestyle and personal hygiene. This is greatly applicable in context of Indian populations owing to the fact that there are vast differences in diet habits, lifestyle and intake of medicines among the communities in spite of a great ethnic diversity (Ahmed *et al*., 2003).

Various typing methods including PCR–based randomly amplified polymorphic DNA fingerprinting (Fantry *et al*., 1996), hybridization with specific probes (Steichen *et al*., 2007) and pulsed field gel electrophoresis (PFGE) have been used to study the high degree of genomic diversity prevailing in *H. pylori*. PCR-RFLP method has also been used for typing the *ureC* gene in order to relate *H. pylori* epidemiologically (Stone *et al*., 1997). Prompted with the usefulness of RFLP technique, we applied this method to study the diversity of *cagA* gene. Accordingly, 3.5 kb amplified product of the *cagA* gene was obtained from all the isolates. Altogether 29 distinguishable RFLP types after combining the restriction pattern of *Hind*III and *Alu*I were obtained from 32 *H. pylori* isolates. Cluster analysis placed all these RFLP types in five groups. Results obtained by this method clearly showed heterogeneity among various *H. pylori* isolates. Till date there is no data on PCR-RFLP types of *cagA* gene based on a large fragment size of 3.5 kb. We feel that PCR-RFLP of *cagA* gene could be conveniently used for the study of genetic diversity among *H. pylori*. Our data are consistent with other reports where genetic diversity of *ureC* in *H. pylori* using the RFLP method has been demonstrated (Stone *et al*., 1997). Genetic diversity in *H. pylori* based on *ure*C gene was further corroborated from an earlier finding which reported that 25 different isolates could be divided into 25 different types when the amplified product of *ureC* gene was digested with *Hha*I, *Mbo*I and *Mse*IA (Fujimoto *et al*., 1994). That the *cagA* typing may indeed be useful in revealing genetic diversity among different strains of *H. pylori* is also evident from the genetic relatedness of various isolates. Our data of cluster analysis revealed that, i) majority of the isolates from Varanasi (North) and Hyderabad (South India) belonged to different groups; ii) strains from both the regions although showing identical clinical outcomes fall under different groups and, iii) out of 32 clinical isolates, two isolates from group D and three from group B shared 100% similarity. These results suggest that

infection by *H. pylori* is not due to a single or few types of strains and more importantly there are variations among isolates of North and South India.

In conclusion, our findings clearly demonstrate diversity in *cagA* gene and differences in the distribution and integrity of *cag* PAI among the *H. pylori* isolates of North and South India. Furthermore RFLP of *cagA* could be conveniently used for genotyping of a large number of strains for obtaining useful information. We feel that data on geographical distribution of *cag* PAI rearrangement patterns may provide hitherto unknown facts related to bacterial virulence, host genetic predisposition, and niche characteristics. It would be desirable to include large number of subjects for the study of genetic diversity among various isolates of *H. pylori* especially on the basis of *cag*A genotyping.

HOST FACTORS



Risk of gastric cancer due to polymorphism of IL-1B and IL-1RN and infection of *H. pylori*

Chapter 4

HOST FACTORS

Interleukin-1B and 1RN Polymorphisms and Their Role (s) in Pathogenesis of Gastric Cancer

1 Introduction

In general it is felt that superficial chronic gastritis progresses to chronic atrophic gastritis over a period of decades (Kekkj *et al*., 1980; Villako and Siuala, 1981). It has been reported that atrophic gastritis is a precursor lesion for gastric cancer (Imai *et al*., 1971; Munoz *et al*., 1968; Strickland *et al*., 1993). The causes of gastric cancer (GC) are not well understood, although nutritional and genetic factors have been suggested in a multifactorial and multistage process (Correa, 1992). El-Omar *et al*. (2000; 2001) reported the first positive association between gastric cancer risk and polymorphisms in the genes coding for the cytokines interleukin 1B (IL-1B) and interleukin 1 receptor antagonist (IL-1RN). These interleukin genes are located on chromosome 2q14, within a 360-kb region (Ruzzo *et al*., 2005). IL-1RN counter balances the potentially injurious pro-inflammatory effects of IL-1B. Functional polymorphisms in IL-1B and IL-1RN have been found to impair this fine interplay, causing an unfavourable high IL-1B/IL-1RN ratio (Vamvakopoulos *et al*., 2002; Witkin *et al*., 2002). IL-1B is not only a phenotype determinant but it also seems to be a major co-factor in promoting and maintaining *H pylori* infection (Rad, *et al*., 2003), which is a recognised etiologic factor for gastric cancer. The association of these variants with gastric cancer has been confirmed in many other populations (Mansour *et al*., 2006; Munoz *et al*., 1968). However little is known about the role of IL-1B and IL-1RN polymorphisms in the development of gastric cancer particularly in Indian context. There are no reports of IL-1B and IL-1RN polymorphism and *H. pylori* active infection in patients of India especially using gastric tissue as study material. This gap of knowledge prompted us to investigate IL 1B −511, IL 1B −31, IL-1B +3954 and IL-1RN 86 variable number tandem repeats (VNTR) polymorphism in *H. pylori* infected patients. The polymorphic site included promoter region of IL-1B at position -511, +3954 (C-T transition), and -31 (T-C transition) and IL-1RN (VNTR).

2 Materials and methods

2.1. Patients

One hundred ten (normal) and one hundred thirty six cases of gastric cancer from the Department of Gastroenterology, S. S. Hospital, Banaras Hindu University, Varanasi, India, were included in this study. The hospital provides health services to a large number of populations of North-eastern India. Details of biopsy collection, case history etc. is mentioned in Chapter 3.

2.2 Analysis of the IL-1B-31, IL-1B-511, IL-1B +3954 and IL1-RN polymorphisms

The IL-1B-511, IL-1B-31, and IL-1B +3954 polymorphisms are single nucleotide changes in the promoter region, while the IL-1RN polymorphism is a 86-base pair VNTR within the second intron. Genotyping and SNP study of IL-1B-511 allele were performed by PCR amplification followed by RFLP. The PCR reaction mix included 0.75 U of *Taq* DNA polymerase (Bangalore Genei), 1 X PCR buffer with 1.5 mM $MgCl_2$, 15 pmole each of the forward and reverse primers (Mansour *et al*., 2006, Zhang *et al*., 2005) (Table 4.1), 100 µM each of the dNTPs and 100 ng of template DNA in a total volume of 25 µl. Thermal cycle for amplification was set as: initial denaturation (94 ^{o}C for 10 min), followed by 5 cycles of denaturation (94 ^{o}C, 30 s), annealing (65 ^{o}C, 30 s) and extension (72 ^{o}C, 30 s); 30 cycles of 94 ^{o}C for 30 s, 60 ^{o}C for 30 s and 72 ^{o}C for 30s; and finally, 5 cycles of 94 ^{o}C for 30 s, 55 ^{o}C for 30 s and 72 ^{o}C for 30 s. Amplified PCR products were digested by *Ava*l (Promega) at 37 ^{o}C for 16 h and then analyzed by 2.5% agarose gel electrophoresis. Alleles were coded as: 155 bp for T/T; 88 and 67 bp for C/C; 155, 88 and 67 bp for C/T as per the earlier report (El-Omar *et al*., 2000).

Genotyping of IL-1B-31 allele was done by PCR amplification in a final volume of 25 µl with primers designed and reported by El-Omar *et al*. (2000). The PCR reaction mix and PCR conditions were same as used for IL-1B-511. PCR products were digested with *Alu*1 (Promega) at 37 ^{o}C for 16 h and analyzed by 2% agarose gel electrophoresis. There were 3 fragments of 239, 137 and 102 bp and grouping of coding alleles C/C (239 bp), T/T (137 and 102 bp), and C/T (239, 137 and 102 bp), was done according to Zhang *et al.* (2005). Genotyping of IL-1B+3954 alleles was done by PCR amplification followed by restriction digestion. The PCR reaction mix was similar to those used for IL-1B-511.

Thermal cycles for the amplification were set as; initial denaturation (94 ^{o}C for 10 min), followed by 5 cycles of denaturation (94 ^{o}C, 30 s), annealing (64 ^{o}C, 30 s) and extension (72 ^{o}C, 30 s); then 30 cycles of 94 ^{o}C for 30 s, 59 ^{o}C for 30 s and 72 ^{o}C for 30s and finally, 5 cycles of 94 ^{o}C for 30 s, 54 ^{o}C for 30 s and 72 ^{o}C for 30 s. The PCR products were digested by *Taq*I (Promega) at 37 ^{o}C for 16 h and then analyzed by 2.5% agarose gel electrophoresis. There were 3 fragments of 249, 135 and 114 bp, the grouping of coding alleles namely, T/T (249 bp), C/C (135 and 114 bp) and C/T (249, 135 and 114 bp) was based as per the earlier report (Zhang *et al*., 2005). Polymorphic study of IL-1RN was also done by PCR assay. PCR reaction mix and thermal cycles were same as IL-1B-511. Amplified PCR products were analyzed by 2% agarose gel electrophoresis. Alleles were coded conventionally as: allele 1=4 repeats, allele 2=2 repeats, allele 3=5 repeats, and allele 4=3 repeats (Zhang *et al*., 2005).

Table 4.1 Primers used for the amplification of IL-1B promoter and repeated intron region of IL-1RN

Primer	Primer Sequences	Product Size (bp)
IL-1B-31 (F)	5'-AGA AGC TTC CAC CAA TAC TC-3'	239
IL-1B-31 (R)	5'-AGC ACC TAG TTG TAA GGA AG-3'	
IL-1B-511 (F)	5'-GCC TGA ACC CTG CAT ACC GT-3'	155
IL-1B-511 (R)	5'-GCC AAT AGC CCT CCC TGT CT-3'	
IL-1B+3954 (F)	5'-GTT GTC ATC AGA CTT TGA CC-3'	249
IL-1B+3954 (R)	5'-TTC AGT TCA TAT GGA CCA GA-3'	
IL-1RN (VNTR) (F)	5'-CCC CTC AGC AAC ACT CC-3'	2-5 repeats
IL-1RN (VNTR) (R)	5'-GGT CAG AAG GGC AGA GA-3'	

Primer sequence adopted from Zhang *et al*. (2005).

2.3 Statistical analysis

Harddy-Weinberg equilibrium of alleles at individual loci was assessed by χ^2 statistics. Genotype frequencies were analyzed by odds ratio and 95% CI using the program SPSS (version 11.5).

3 Results

3.1 Test for polymorphism in interleukin 1B and 1RN genes

Typical representation of PCR-RFLP of IL-1B-51, -31, +3954 and VNTR of IL-1RN is represented in Fig. 4.1 A, B, C and D. Our results of interleukin 1B and 1RN genes polymorphism are based on 136 cases and 110 controls. The mean age of the cases was 65.4 ± 5.2 and that of controls was 42.5 ±7.1. Eighty five (62.5%) of the cases was males and fifty one (37.5%) female. Eighty four (76.36%) of the controls were males and twenty six (23.64%) female. The frequencies of the tested genotypes were in accordance with Hardy - Weinberg equilibrium. The frequency of IL-1RN 2/2 was significantly higher in GC case (21.32%) than the controls (9.09%) with an Odds Ratio (OR) of 4.39 (95% CI 1.90-10.13) (Table 4.2). The IL-1RN 1/2 genotype in the case group had a frequency of 50% compared to 42.73% in control with an OR of 2.19 (95% CI 1.24-3.86). The IL-1B-31 C/C genotype showed significantly higher frequency in cases (41.18%) than the controls (30%) with an OR of 2.00 (95% CI 1.03-3.88). The frequency of IL-1B-511 T/T was also higher in cases (35.29%) in comparison to the controls (22.73%) with an OR of 1.99 (95% CI 0.98-4.01). The IL-1B+3954 C/T genotype was relatively higher in cases (28.68%) than the controls (20.90%) with an OR of 1.66 (95% CI 0.96-3.00) (Table 4.2).

3.2 Interleukin 1B and 1RN genes polymorphism and *H. pylori* infection

We have analyzed the association between gastric cancer risks and its relation with *H. pylori* active infection. Evaluation was made using non – infected (*H. pylori*$^-$) case of C/C genotype of IL-1B-511 gene as the reference group (Table 4.3).

It is evident from the results of Table 4.3 that C/T genotype of IL-1B-511 although had higher odds ratio (5.87, 95% CI =0.70-49.38) but significance was on the border line (p=0.067). In the case of T/T genotype the odds ratio increased further (13.00, 95%CI = 1.42-119.07) and the value was moderately significant (p=0.010). However in the presence of *H. pylori* infection odds ratio showed marked increase in C/C, C/T and T/T genotypes, the χ^2 being 12.97, 15.89 and 18.98 for the above genotypes respectively. Analysis of data clearly suggests that IL-1B-511 genotype has relatively increased risk of developing gastric cancer (p<0.001) in comparison to non-infected patients.

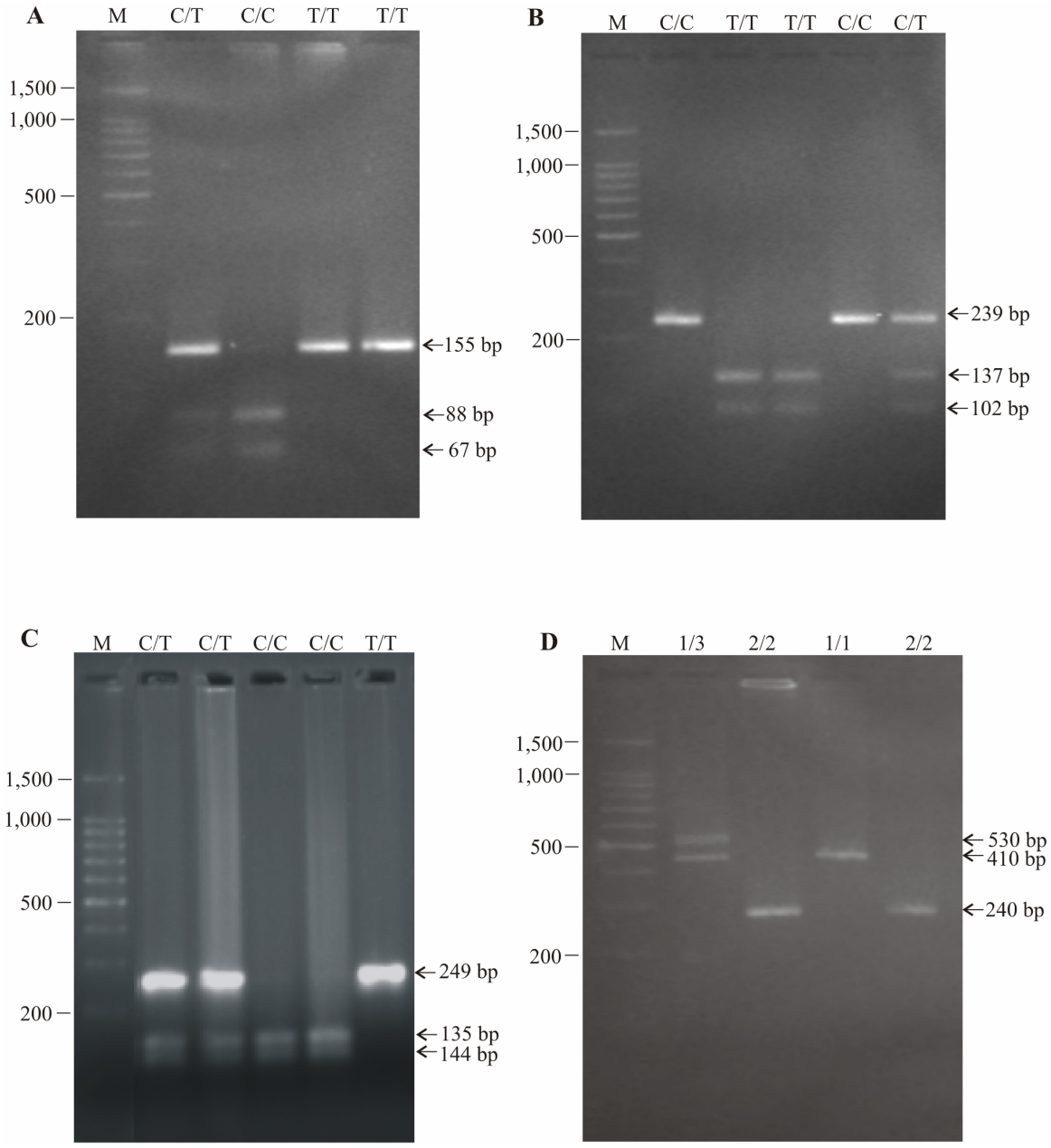


Fig. 4.1 A typical representation of genotyping of IL-1B and IL-1RN variable number of tandem repeats polymorphisms. (A) PCR-RFLP of the IL-1B-511 polymorphism showing; M- 100bp ladder, C/C, C/T, and T/T. (B) PCR-RFLP of the IL-1B-31 polymorphism showing the M- 100 bp ladder, C/C, T/T, and C/T. (C) PCR-RFLP of the IL-1B+3954 polymorphism: M- 100 bp ladder, C/T, C/C and T/T, and (D) Agarose gel electrophoresis of the IL-1RN VNTR polymorphism showing ; M- 100 bp ladder, 1/3, 2/2, 1/1, and 2/2.

Table 4.2 IL-1B and IL-1RN genotype frequencies in gastric cancer case and control in North Indian populations

Locus	Genotype	Case n=136 (%)	Control n=110 (%)	Odds Ratio (95% CI)
IL-1B-511	C/C (ref)	29 (20.59)	30 (27.27)	-
	C/T	59 (44.12)	55 (50.00)	1.11 (0.59-2.08)
	T/T	48 (35.29)	25 (22.73)	1.99 (0.98-4.01)
	CT /TT	107 (79.41)	80 (72.73)	1.4 (0.78-2.51)
IL-1B-31	T/T (ref)	28 (20.59)	33 (30.00)	-
	C/T	52 (38.23)	44 (40.00)	1.39 (0.73-2.65)
	C/C	56 (41.18)	33 (30.00)	2.00 (1.03-3.88)
	CT /CC	108 (79.41)	77 (70.00)	1.65(0.92-2.96)
IL-1B+3954	C/C (ref)	89 (65.44)	87 (79.09)	-
	C/T	39 (28.68)	23 (20.90)	1.66(0.96-3.00)
	T/T	8 (5.88)	0	Infinite
	CT /TT	47 (34.56)	23 (20.90)	1.91 (1.07-3.47)
IL-1RN	1/1 (ref)	35 (25.74)	53 (48.18)	-
	1/2	68 (50.00)	47 (42.73)	2.19 (1.24-3.86)
	2/2	29 (21.32)	10 (9.09)	4.39 (1.90-10.13)
	1/3	4 (2.94)	0	Infinite
	1*2*/2*2*	97 (71.32)	57 (51.82)	2.58 (1.51-4.41)

Analysis of IL-1B-31 was done using T/T genotype from non–infected patient as the reference group. It is evident from the results that the C/T genotype in the absence of *H. pylori* infection has an odds ratio of 2.93, 95%CI =0.70-12.33 but was insignificant (p=0.118). C/C genotype had slightly higher odds ratio (3.26, 95% CI =0.77-13.80) but was insignificant (p=0.091). On the other hand the odds ratio of the genotypes T/T, C/T and C/C increased significantly with *H. pylori* infection and the value of χ^2 also showed increasing trend with increase in odds ratio (Table 4.3). It is also evident from the data that the p value is <0.001 in all the genotypes suggesting that the risk of developing cancer is more prominent in the presence of *H. pylori*.

Table 4.3 Odds ratio and risk of developing gastric cancer on the basis of IL-1B-511, -31, IL-1B+3954 and IL-1RN genotypes following *H. pylori* infection in North Indian populations

Locus and *H. pylori* staus	Genotype	Case n=136	Control n=110	Odd Ratio (95% CI)	χ^2	[a]p value
IL-1B-511						
H. pylori$^-$	C/C	1	13	-		
	C/T	14	31	5.87 (0.70-49.38)	3.24	0.067
	T/T	10	10	13.00 (1.42-119.07)	6.91	0.01
H. pylori$^+$	C/C	28	17	21.41 (2.57-178.63)	12.97	0.0003
	C/T	45	24	24.38 (3.01-197.74)	15.89	<0.001
	T/T	38	15	32.93 (3.953-274.36)	18.98	<0.001
IL-1B-31						
H. pylori$^-$	T/T	3	16	-		
	C/T	11	20	2.93 (0.70-12.33)	2.27	0.118
	C/C	11	18	3.26 (0.77-13.80)	2.724	0.091
H. pylori$^+$	T/T	25	17	7.843 (1.98-31.13)	10.08	<0.001
	C/T	41	24	9.111 (2.41-34.52)	13.18	0.001
	C/C	45	15	16.000 (4.09-62.62)	21.22	<0.001
IL-1B +3954						
H. pylori$^-$	C/C	15	42	-		
	C/T	9	12	2.10 (0.74-5.98)	1.97	0.13
	T/T	1	0	infinite	2.67	0.276
H. pylori$^+$	C/C	74	45	4.604 (2.30-9.24)	19.84	<0.001
	C/T	30	11	7.64 (3.08-18.94)	21.08	<0.001
	T/T	7	0	0	15.01	-
IL-1RN						
H. pylori$^-$	1/1	4	27	-		
	1/2	13	25	3.51(1.01-12.20	4.17	0.037
	2/2	0	2	0	-	-
H. pylori$^+$	1/1	31	26	8.05(2.49-25.99)	14.43	<0.001
	1/2	55	22	16.88 (5.29-53.87)	30.54	<0.001
	2/2	21	8	17.72 (4.69-66.92)	21.83	<0.001
	1/3	4	0	infinite	-	-

[a]Fisher's Exact Test -Exact Sig. (1-sided)

In the case of IL-1B+3954, analysis was made using C/C genotype as the reference from the non-infected case. Results showed that C/T had an odds ratio of 2.10, 95%CI =0.74-5.98, the value being not significant (p=0.130) (Table 4.3). On the other hand in the presence of *H. pylori* infection, both C/C and C/T had higher values of odds ratio and χ^2 ($p<0.001$) suggesting an increased risk of gastric cancer. Analysis of IL-1RN using non – infected patient and 1/1 as reference also showed increased values of odds ratio in 1/1, 1/2 and 2/2 genotypes in the presence of *H. pylori* infection suggesting an increased risk of GC ($p<0.001$).

4 Discussion

Inflammation in the form of chronic superficial gastritis is thought to be one of the early phases in the development of intestinal-type gastric cancer (Correa *et al*., 1992), the predominant histologic type of gastric cancer. Therefore, a stronger inflammatory response by the host could theoretically modify gastric cancer risk. The first published epidemiologic study examining such associations found that proinflammatory polymorphisms in IL-1B and IL-1RN were strongly associated with both gastric cancer and chronic atrophic gastritis (EI-Omar *et al*., 2001), a precursor of gastric cancer. Further evidence came from a subsequent study that showed >20-fold increased risk of gastric cancer associated with the presence of three or more proinflammatory polymorphisms in IL-1B, IL-1RN, IL-10, and TNF-A (EI-Omar *et al*., 2003).

Biological evidence for and against the above-mentioned associations are also mixed. El-Omar (2001) has given a comprehensive review of the biological effects of IL-1β. According to his study, on the one hand, proinflammatory polymorphisms in IL-1B and IL-1RN may reduce gastric cancer risk by mounting a stronger inflammatory reaction against *H. pylori*, reducing gastric injury in response to a wide variety of noxious stimuli, and increasing apoptosis of gastric epithelial cells. On the other hand, stronger inflammatory reaction may increase cancer risk by causing genomic damage to gastric cells, mucosal atrophy, and secondary hypochlorhydria and bacterial overgrowth (EI-Omar, 2001).

H. pylori-associated disease depends on *H. pylori* infection and the host genetic background, which is reflected by disease outcome. Among the host determinants of *H. pylori*-associated disease outcome, the balance between pro-inflammatory and anti-inflammatory cytokines seems to play a major role. Genetic polymorphisms of host's

cytokine genes might alter the amount of cytokines produced and released in response to *H. pylori* infection (El-Omar *et al*., 2000, 2001). In the present investigation an attempt was made to test the role of interleukin genes polymorphisms in disease outcome both in the presence and absence of *H. pylori* infection. To our knowledge studies in this context have been made using blood for genotyping as well as for passive infection of *H. pylori*. We have used gastric tissue obtained from endoscopy for assessing the role of different genotypes in the disease outcome. The findings of the present study suggest the role of *H. pylori* active infection and SNP of promoter region of -511, -31 and +3954 of IL-1B and IL-1RN VNTR polymorphism in gastric cancer.

In this study, we have made an attempt to reveal association, if any, between different IL-1B and IL-1RN genotypes and frequencies of GC cases. From our finding it is evident that IL-1RN 2/2 is associated with significantly increased risk of GC. Our results are in agreement with Omani Arab population (Mansour *et al*., 2006). Additionally our results also show that the genotypes IL-1RN 1/2, IL-1B-31 C/C, IL-1B-511 T/T and IL-1B+3954 C/T have higher tendency to GC. Partly our observation resembles to the studies made by El- Omar *et al.* (2000-2001) who reported that IL-1B-31C+ and IL-1RN 2/2 genotypes were associated with increased risk of GC in Caucasians. A study made in China has also reported that IL-1B +3954 C/T heterozygote was associated with increased risk of GC (Zhang *et al*., 2005). They reported that IL-1B-511 C/T had higher susceptibility to GC in Chinese population. However our results show that IL-1B-511 T/T has higher susceptibility than IL-1B -511 C/T.

We have also studied the interaction between *H. pylori* infection and IL-1B and IL-RN genotypes polymorphism. In a Japanese study, Takaji *et al*. (2002) found that IL-1B polymorphism (IL-1B-511C/C and -31T/T) enhanced not only IL-1ß production but also caused IL-8 production in the gastric body. They suggested that the above genotypes were associated with *H. pylori* infected gastric mucosal lesions. Similar to their observations our study also showed that IL-1B-511 C/C and IL-1B-31 T/T genotypes caused significant increase in risk of GC when infected with *H. pylori.* We noted that the *H. pylori* was present in 81.6% of cases and 50% of controls. In contrast Saxena *et al.* (2008) from Lucknow, India, reported that the *H. pylori* was present in 55.2% of controls (NUD) and 56.5% in the cancer cases. As such their data was not statistically significant as well as they used ELISA (passive infection) test for the detection of *H. pylori*. Most probably differences in the prevalence of *H. pylori* might be due to the method of

detection since we employed PCR assay (active infection) which is more sensitive technique for bacterial detection. In general it is felt that the amount of antibody production is subject to the host immunity. If so it is possible that the antibody production in GC patients in Indian subjects is low. Sivprakash, *et al*. (1996) from Madras, India, have also reported that the prevalence of *H. pylori* by various tests ranged from 56.0 to 62.6% in the gastric carcinoma cases and 37.3 to 46.6% in the control subjects, the difference being statistically significant. In the present investigation we have observed that individuals with both *H. pylori* infection and IL-1B variant genotypes had an increased risk of GC in comparison to those having IL-1B variant genotype only, indicating a potential gene- environment interaction. It is presumed that the association of these SNPs with GC may require the presence of *H. pylori* in gastric carcinogenesis, a fact which is consistent with the effects of biological interaction between *H. pylori* infection and IL-1ß secretion. When *H. pylori* infection challenges the gastric mucosa, a vigorous inflammatory response with a high IL-1ß expression may be beneficial for the eradication of *H. pylori*, but the accompanied inhibition of acid secretion may extend the area of colonization, resulting in inflammation in the corpus mucosa, and may cause early onset of gastric atrophy and malignant transformation. In conclusion our results clearly show that interleukin-1B and 1RN polymorphisms of the host indeed play important role in the development of GC.

Summary

The present study deals with isolation of *H. pylori* from patients suffering from various types of gastrointestinal diseases and characterization and genotyping of certain genes involved in disease appearance. Attempts were also made to reveal the role of environmental and host factors in the disease outcome. All the studies were conducted in collaboration with the Department of Gastroenterology, Institute of Medical Sciences, which is associated with S. S. Hospital, Banaras Hindu University, Varanasi, India. The Hospital provides territory level health care to large number of persons. The study involved 276 cases from whom biopsy samples (three biopsies from each patient) and detailed history of complications were obtained. With a view to screen the presence of *H. pylori*, URUT was done with fresh biopsy samples of all the patients. Of the 276 patients, URUT gave positive test in biopsy of 221. Results of URUT were further confirmed by the amplification of *H. pylori* specific 16S rDNA. Results of 16S rDNA amplification revealed the presence of *H. pylori* in 226 (81.88%) cases comprising 54 gastritis, 86 ulcer, 52 cancer and 34 normal patients. Survey of patients based on disease history, food habits, number of family members etc showed that the prevalence of *H. pylori* increased till 31 to 40 years age group and thereafter decreased. Additionally positive association with factors like number of family members, sources of drinking water and dental habits was a noteworthy finding. Interestingly prevalence of *H. pylori* was negatively associated with factors like smoking, intake of citrus fruits and no correlation was observed with exercise habits and schedule of taking food.

A novel multiplex PCR assay for the diagnosis as well as characterization of important pathogenicity genes was developed. This method does not require boiling of biopsy sample and/or repeated centrifugation; simply biopsy samples are vortexed and the small pieces of tissues present, if any, in the suspension are removed, the resulting suspension (devoid of tissues) is used as a source of template. Template obtained by the above method showed excellent amplification of *H. pylori* specific 16S rDNA in 226 patients (out of 276). Identical result was obtained when multiplex PCR assay was performed with the template used above (obtained by vortexing of biopsy tissues from 226 patients). Further analysis of data based on both URUT and PCR assay showed the presence of *H. pylori* in 217 patients and their absence in 46 patients (n= 263, out of 276). In the remaining 13 samples, 9 was positive with PCR assay and 4 with URUT

only. Evidently our results showed a sensitivity of 96% and specificity of 92% when the results of multiplex PCR assay were compared with URUT. Out of 226 *H. pylori* positive biopsy samples, we could successfully isolate and grow culture of *H. pylori* from 198 samples and all showed amplification of desired amplicons of *H. pylori*. Further analysis revealed that out of 276 patients, 36 had multiple *H. pylori* strain infection and 8 (5 gastritis patients and 3 normal cases) showed the amplification of 16S rDNA only (*cagA*$^-$ and *vacA*$^-$).Unless otherwise stated 232 patients were chosen for further study. Genotypic profile of *H. pylori* from all the 182 patients (excluding 50 *H. pylori* negative patients) was analysed. Form the data it was evident that the prevalence of *H. pylori* was highest in ulcer followed by gastritis and cancer patients. Of the 182 patients suffering from single strain infection, s1 genotype of *vacA* was detected in all the gastritis and cancer patients whereas the number was less in ulcer (87.1%) and normal cases (82.1%). Overall 92.3% patients had s1 genotype and 7.7% had s2 genotype. The *vacA* m1 genotype was detected in (58.2%) patients, the highest being in gastritis followed by ulcer patients. *vacA* m2 genotype was highest in normal (75.0%) followed by cancer patients (43.2%). Further analysis of *vacA* gene revealed that s1/m1 combination was more common since 58.2% patients carried this genotype while s1/m2 genotype was present in 34.1%. Notably the frequency of s2/m2 genotype was smaller (7.7%) and s2/m1 genotype was at all not present in any patient. 36 patients who had mixed infections exhibited different combination of both s1 and s2 of the signal region and m1 and m2 from the middle region. Evidently our results demonstrate the possible occurrence of multiple *H. pylori* strain infection in different patients. *cagA* was detected in 115 (62.3%) patients and was present in 100 % cancer, 70% gastritis, 54.3% ulcer patients, and in 17.9% normal cases. It was also noted that *cagA* was strongly associated with s1/m1 genotype followed by s1/m2 and least with s2/m2.

36 *H. pylori* isolates comprising 22 from Hyderabad (South India) and 14 from Varanasi (North India) were selected to study genetic diversity. Among the 14 patients of Varanasi, 4 had gastritis, 3 GC, 2 DU, 1 GU and 4 was normal. Among Hyderabad patients, 13 had DU, 4 GU, 2 GC, 2 gastritis and 1 was normal. With a view to confirm the presence of *H. plyori* and to deduce phylogenetic relatedness, 16S rDNA from 11 isolates of Hyderabad, 14 from Varanasi and pathogenicity genes (*cagA*, *cagE*, *cagT*, *vacA* s1, *vacA* s2, *vacA* m1, *vacA* m2, *iceA*1 and *iceA*2) of selected Varanasi isolates were amplified and sequenced. Sequence analysis showed 97-99% homology with the

sequences of *H. pylori* available in the database. All the 16S rDNA and pathogenicity genes sequences were submitted to NCBI and accession numbers were obtained (Accession no of Varanasi strains: FJ200229 to FJ200241, FJ984535 to FJ984548 and Hyderabad strains: DQ912962 to DQ912970). A phylogenetic tree based on 16S rDNA of 184 taxa including 160 reference strains from the data base and 24 strains of the present study was generated. Analysis revealed that strains from Hyderabad and Varanasi clustered separately and showed significant diversity. Interestingly, Varanasi isolates showed close homology to the isolates of Tiawan whereas Hyderabad isolates shared homology with the strains of Brazil.

Molecular diversity among 36 isolates (as mentioned above) was studied by ARDRA, following restriction digestion with two tetra-cutter restriction enzymes, *Alu*I and *Rsa*I, however the results did not show significant diversity among these isolates. On the other hand ERIC-PCR assay revealed some diversity among all these isolates. To have a better understanding of diversity, amplified fragment length polymorphism (AFLP) was tested. Accordingly whole genome of selected isolates was digested with *Hind*III and ligated with linker DNA and finally amplification was done using HI-A and HI-C primers for the analysis of AFLP pattern. Amplification with HI-A showed 5-12 bands whereas HI-C showed 2-8 bands. Evidently distinguishable banding pattern by both the primers were observed among all the isolates. A phylogenetic tree based on the AFLP pattern using UPGMA was constructed so as to reveal genetic relationship among 36 clinical isolates together with standard ATCC reference strains (60190 and 26695). Similarity coefficient revealed that all the 38 strains could be placed into 3 major groups. Analysis showed that there was distinct diversity between North and South Indian isolates. Interestingly not a single isolate showed 100% similarity with each other. Our results clearly demonstrated that inter strain genomic heterogeneity is indeed a characteristic feature of *H. pylori***.**

Further diversity among all the 36 isolates of *H. pylori* was made targeting *cag* PAI which contains a cascade of important genes. To achieve this objective, five pairs of primers were used to detect the presence of the *cagA*, *cagE* and *cagT* genes. Three sets of primers were used to detect the *cagA* gene, two for 5' and one for 3' end. For elucidating the presence of *cagE* and *cagT*, one set of primer was used for each gene. *H. pylori* strain ATCC 60190, which contains the entire *cag* PAI region served as positive

control for each PCR assay. Distribution pattern of *cagA* revealed that out of 22 strains of Hyderabad, *cagA* 5' region was present in 95.4% strains and *cagA* 3' region was present in all the strains. Among the isolates of Varanasi, 78.57% showed the presence of *cagA* 5' and 92.85% *cagA* 3'. Additionally, out of 22 strains isolated from the patients of Hyderabad, 9% showed partial deletion and the remaining 91% had intact *cag* PAI. On the other hand, among the strains of Varanasi, 35.7% showed partial deletion and 64.3% contained intact *cag* PAI. Interestingly none of the strains either from Hyderabad or Varanasi exhibited complete deletion of *cag* PAI. With a view to examine variations in the *cagA* variable region, PCR amplification of *cagA* 3' variable region was performed and size of all the products was determined. The size of PCR products in all the isolates ranged from ca. 1042 to 1360 bp. Among the strains belonging to Varanasi region, the size was in the range of 1042 to 1267 bp whereas it was in the range of 1167 to 1360 bp in the strains of Hyderabad. Analysis of *cagA* diversity among various strains of *H. pylori* was performed by the amplification of approx. 3.5 kb *cagA* gene using forward primer for *cagA*-5' end and reverse primer for *cagA*-3' end. Amplification was successfully achieved in 95.45% and 78.57% strains of Hyderabad and Varanasi respectively. Amplified product was digested by two restriction enzymes, namely *Hind*III and *Alu*I separately. Results showed 29 distinguishable RFLP types from 32 isolates. A dendrogram based on the RFLP pattern of *cagA* was constructed using UPGMA to reveal genetic relationship among 32 clinical isolates together with standard ATCC reference strain (60190). Similarity coefficient revealed that all the 32 strains could be placed in 5 major groups, namely A, B, C, D and E. This result also revealed diversity in *cagA* among isolates of distinctly located regions.

With a view to study the role of host genetic factors, analysis was made covering 136 cases and 110 controls. The mean age of the cases was 65.4 ± 5.2 and that of controls were 42.5 ±7.1. All the frequencies of the tested genotype were in accordance with Hardy - Weinberg equilibrium. The frequency of cases in IL-1RN 2/2 was significantly higher in GC case group than in the controls with an Odds Ratio (OR) of 4.39. The case group of IL-1RN 1/2 genotype had a frequency of 50% compared to 42.73% in control group with an OR of 2.19. The IL-1B-31 C/C genotype showed significantly higher frequency in cases than in controls with an OR of 2.00. The frequencies of cases in IL-1B-511 T/T were significantly higher in cases compared to the controls with an OR of 1.99. The IL-1B+3954 C/T had a significantly higher frequency in cases compared to the controls with

an OR of 1.66. We have analyzed the association between gastric cancer risks in relation to *H. pylori* active infection. The genotypes were studied in detail and its association with the *H. pylori* active infection was examined. Our results showed that IL-1B -511 C/C and IL-1B -31 TT genotypes caused significant increase in the risks of GC when infected with *H. pylori.* Altogether from the findings of our study it appears that interleukin 1B and 1RN polymorphisms of the host do play important role in the development of GC.

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Publications from the Thesis

Environmental Factors

Kumar, S., Kumar, A. and Dixit, V. K. Role of Environmental Factors in Gastrointestinal Diseases: Relation to Food Habit and Mode of Living (To be communicated).

Pathogen Factors

Kumar S., Kumar, A. and Dixit V. K. (2008). Direct detection and analysis of *vacA* genotypes and *cagA* gene of *Helicobacter pylori* from gastric biopsies by a novel multiplex polymerase chain reaction assay. **Diagnostic Microbiology and Infectious Disease 62** (4): 366–373.

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Host Factors

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Abstract

Kumar, S., Kumar, A. and Dixit, V. K. (2007). **Genetic Diversity in *Helicobacter pylori* in North India**. In: National Conference on "Microbial Diversity Avenues and Application", Division of Life Science, Sardar Bhagwan Singh Post Graduate Institute of Biomedical Science and Research, Dehradun, Utrakhand, India.