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Full Length Research Paper

Analysis of *CagA*, *VacA* and *IceA* genotypes of colonized *Helicobacter pylori* and Interleukin-1 receptor antagonist (*IL-1RN*) gene polymorphism among dyspepsia patients

Suneesh Kumar Pachathundikandi[1]*, Anish Kumar[2], Prakash Zacharias[2] and Joseph Madassery[1]

[1*]Department of Biotechnology, University of Calicut, Calicut University (P O), Kerala -673635, India
[2]Department of Gastroenterology, Malabar Institute of Medical Sciences, Kozhikkode, Kerala-673016, India

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Pro-inflammatory Interleukin-1 receptor antagonist (*IL-1RN*) gene polymorphism and high risk *Helicobacter pylori* virulence factors in combination plays an important role in the outcome of *H. pylori* associated diseases, which can even lead to gastric cancer. The purpose of this study was to analyze the prevalence of high risk host and bacterial factors such as *IL-1RN* gene variable number tandem repeat (VNTR) polymorphism and *cagA, vacA* and *iceA* genotypes among a group of *H. pylori* infected dyspepsia patients from Kozhikode area of Kerala state, India. Mucosal biopsies from fourty two randomly selected dyspepsia patients infected with *H. pylori,* as determined by rapid urease test (RUT) and *glmM* gene specific PCR, were used in this study. The high risk bacterial virulence factors such as *vacA,cagA,iceA1* and *iceA2* genotype status of colonized *H. pylori* and the high risk host pro-inflammatory *IL-1RN*2/2 allele status were examined by PCR methods. The high risk *vacA*s1/*cagA*/*IL-1RN*2/*2* genotype combination was found in six patients (14%), *vacA* s1/*cagA/IL-1RN*1/*1* in three patients (7%) and *vacA*s1/*cagA/IL-1RN*1/*2* in seven patients (16%). In addition, analysis of allelic variation of *iceA* gene of *H. pylori*, such as *iceA1* and *iceA2* genotype, in colonized patients has revealed the co-existence of both *iceA1* and *iceA2* strains among 31% of patients included in this study.**

Keywords: *Helicobacter pylori*, virulence genes, host gene polymorphism, interleukin-1 receptor antagonist.

INTRODUCTION

Over half of the world's population is infected with *Helicobacter pylori*, with the highest rates in developing countries (Rothenbacher and Brenner, 2003). Recent reports are showing that this division is becoming less clear due to the rapid rise of socioeconomic level in subpopulations in many developing countries and to the increasing use of antimicrobials for infection with *H. pylori* and other bacteria in both developed and developing countries (Bruce and Maaroos, 2008).

Infections occur in early childhood and persist for decades in the absence of targeted antimicrobial therapy. In developing world childhood prevalence is more than developed country children. However, the colonization of *H. pylori* with increasing age increased in developed countries. Within geographical areas, the prevalence of *H. pylori* inversely correlates with socioeconomic status, in particular in relation to living conditions during childhood (Perez-Perez et al., 2004). Gastric mucosa is well protected against bacterial infections but *H. pylori* is highly adapted to this ecological niche, with a unique array of virulence factors that permit the attachment of this bacteria to epithelial cells, evasion of the immune

*Corresponding author email: suneeshpt@gmail.com.

response and persistent colonization (Suerbaum and Michetti, 2002). Entry of *H. pylori* in stomach leads to long-term colonization of gastric mucosa and thereby increasing the risk for inflammation and other related pathologies.

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 even gastric cancer (Kusters et al., 2006). Among a large proportion of infected patients *H. pylori* is not causing any severe pathology for the whole period and it is not clear how they are wading the effect of bacterial virulence factors. In addition to bacterial virulence factors, host genetic system, environment and diet have also been implicated for this discrepancy (Matysiak-Budnik and Mégraud, 2006). Inflammatory cytokines and their regulatory components are important in the immunity against microbial infections, this could also been considered for such differences. Genetic polymorphisms of the host can directly affect the expression levels of gene products by generation or deletion of transcription factor sites or by affecting RNA splicing and subsequent translation. Many of the pathogenic effects of *H. pylori* infection are related to chronic active inflammation, which is controlled and maintained by the complex interplay of proinflammatory and anti-inflammatory mediators (Macarthur et al., 2004).

IL-1 Receptor Antagonist (IL-1R,N) is structurally related to IL-1 (Carter et al., 1990; Eisenberg et al., 1990; Eisenberg et al., 1991) but specifically blocks the binding of IL-1α and IL-1β to cell surface receptors without activating target cells (Dripps et al.,1991; Arend et al., 1998). IL-1 stimulates the synthesis of IL-8, which is a potent chemotactic agent for neutrophils, and it also induces release of neutrophil elastase. IL-1 promotes the adhesion of neutrophils and other cells by enhancing the expression of adhesion molecules such as ICAM-1, VCAM-1, and L-selectin (Abdelaziz et al., 1995; Tsang et al., 1997). The *IL-1RN* gene showing polymorphism by penta allelic 86 bp tandem repeat in intron 2, of which allele 2 (*IL-1RN*2/*2*) associated with enhanced IL-1 β production and proinflammatory responses more severe and more prolonged than those of other *IL-1RN* genotypes (Hurme and Helminen,1998; Hwang *et al.,*2002). *IL-1RN*2/*2* (two tandem repeats) homozygous individuals are a distinct minority in every population examined to date. Among the population, most of them are either homozygous *IL-1RN*1/*1* (four tandem repeats) or heterozygous *IL-1RN*1/*2* (Tarlow et al., 1993; Bioque et al., 1995; Jeremias et al., 2000). *IL-1RN*2/*2* genotype may be beneficial in the immune defense against infection by promoting a prolonged Th1 cell-mediated immune response. The T helper cell response towards *H. pylori* is generally considered to be of the Th1 phenotype, leading to a cell-mediated immune response (Shimoyama and Crabtree, 1998; Ernst, 1999). But prolonged Th1 cell mediated immunity is a causing factor for inflammatory conditions and having more evidence that *H. pylori* induced Th1 response contributes to cancer development (Ernst and Gold, 2000).

H. pylori infection shows a heterogeneous picture of pathologies and different involvement among populations and about 20% of those infected ultimately suffer diseases ranging from gastritis and ulcer to gastric cancer. The host specific factors involved in the prolonged colonization and inflammatory conditions with respect to bacterial virulence factors have not been studied in detail among different population for interpreting these discrepancies. *H. pylori* induced hypochlorhydria may be mediated, at least in part, by the proinflammatory cytokine interleukin IL-1β, which is up regulated during chronic *H. pylori* infection (Noach et al., 1994; Peek et al., 1995; Yamaoka et al., 1996). Persistent infection of *H. pylori* causes the inflammation of gastric mucosa and may leads to development of ulcer, MALT lymphoma or cancer. Virulence and pathogenecity factors such *cag* pathogenecity island (*cag*PAI), vacoulating cytotoxin gene-A (*vacA*), and Induced upon contact with epithelium gene-A (*iceA*) are well implicated in studies for their role in the progress of diseases leading to gastric ulcer and gastric cancer (Van Doorn et al., 1998; Peek et al., 1998; Ito et al., 2000). A considerable proportion of patients are not developing severe pathologies in the presence of high-risk bacterial virulence factors. This is undoubtedly pointing the role of host specific factors in the development and severity of disease. A synergistic effect of bacterial virulence factors and host *IL-1RN* and *IL-1* polymorphisms on the development of pre-cancerous lesions and disease development has been reported among patients from Germany (Rad et al., 2003).

Analysis of these high risk factors will help to identify and treat patients carefully and can prevent progression to cancerous lesions. Lacking of data of *H. pylori* colonization and host pro-inflammatory gene polymorphisms among dyspepsia patients from Kozhikode area of Kerala state, India, prompted us to carry out this study. In this study we analyzed the prevalence of *IL-1RN* polymorphism and *cagA, vacA* and *iceA* genotypes among *H. pylori* infected dyspepsia patients from this area.

MATERIALS AND METHODS

Clinical specimen

Gastric mucosal biopsies taken from patients during diagnostic upper intestinal endoscopy in the Department of Gastroenterology, Malabar Institute of Medical Sciences, Kozhikode, Kerala were used for the present investigation (Approved by the Institutional Ethics committee). Rapid urease test and *glmM* gene specific polymerase chain reaction were used for determining *H.*

Table1. Oligonucleotide primers used for analyzing *H. pylori* genotype and VNTR polymorphism of host *IL-1RN* gene.

Human gene		Nucleotide Sequence	Annealing Temperature (ºC)	Reference
IL-1RN	Forward	5' CTCAGCAACACTCCTAT 3'	60	Joos et al., 2001
	Reverse	5' TCCTGGTCTGCAGGTAA 3'		
***H. pylori* Gene**				
glmM	Forward	5' AAGCTTTTAGGGGTGTTAGGGGTTT 3'	54	Bickley et al., 1993
	Reverse	5' AAGCTTACTTTCTAACACTAACGC 3'		
cagA	Forward	5' GATAACAGGCAAGCTTTTGAGG 3'	60	NCBI database
	Reverse	5' CCATGAACTTTTGATCCGTTCGG 3'		
vacA	Forward	5' ACAAACACACCGCAAAATCA 3'	55	NCBI database
	Reverse	5' CCTCTGCCTGCTTGAA 3'		
iceA1	Forward	5' GGATTGCAGCTAGGTGTTCC 3'	54	NCBI database
	Reverse	5' ACCCATCACCATAGCCTTTT 3'		
iceA2	Forward	5' TGCTGCTGTTACCACAAAGG 3'	54	NCBI database
	Reverse	5' CAAGTCTTAACCCCCAACGA 3'		

pylori colonization in these samples. 42 samples infected with *H pylori* as determined by above methods were included for further study.

Rapid urease test (RUT)

Rapid urease solution was prepared using K2HPO4 (11 mM), NaCl (85 mM), urea (0.33 M) and phenol red (100 µM) (Merck, India) in water and pH was adjusted to 6.8. It was then filter (0.2 µm) sterilized and stored at 4ºC. Small piece of gastric mucosal biopsy specimen from patients were incubated in 0.5 ml of the medium in a test tube and positive reaction reported by change in colour from golden yellow to pink within 6 hr and such specimens only used for the present study.

DNA isolation

DNA was extracted from biopsies by boiling in 100 µl of sterile double distilled water for 10 min and cooling it on ice for 5 min. It was then centrifuged at 10,000 rpm for 10 min. The supernatant containing DNA was used of PCR study.

Genotyping of *H. pylori*

PCR was performed using 5 µl of DNA solution at a final volume of 25 µl containing 60mM Tris-HCl, 1.5 mM $MgCl_2$, 25 mM KCl, 250 µM dNTPs, 20 pM each of forward and reverse primers and 1 U of *Taq* DNA polymerase (Biogene, USA) in a MJ Ressearch PTC 150 thermocycler. Oligonucleotide primers for *glmM*, *cagA, vacA* signal region and *iceA1* and *iceA2* (IDT, USA and Genei, India) given in the Table 1 were used for this study. Amplified products were separated by agarose gel (1.5%) electrophoresis and visualized using ethidium bromide staining in a digital gel documentation system (Alpha Imager 2200, USA).

PCR for *IL-1RN* gene VNTR polymorphism

PCR reaction was performed as mentioned in the above case. The following primers flanking polymorphic VNTR region were used for PCR amplification: forward primer 5' CTCAGCAACACTCCTAT 3' and reverse primer 5' TCCTGGTCTGCAGGTAA 3' (Genei, India). Polymerase chain reaction conditions were as follows: 40 cycles at 94°C for 60 s, 60°C for 60 s, and 72°C for 45 s.

RESULTS

***vacA* Signal region and cagA genotypes of *H. pylori* colonized in gastric mucosa of dyspepsia patients**

Genotyping of *vacA* signal region were done using 42

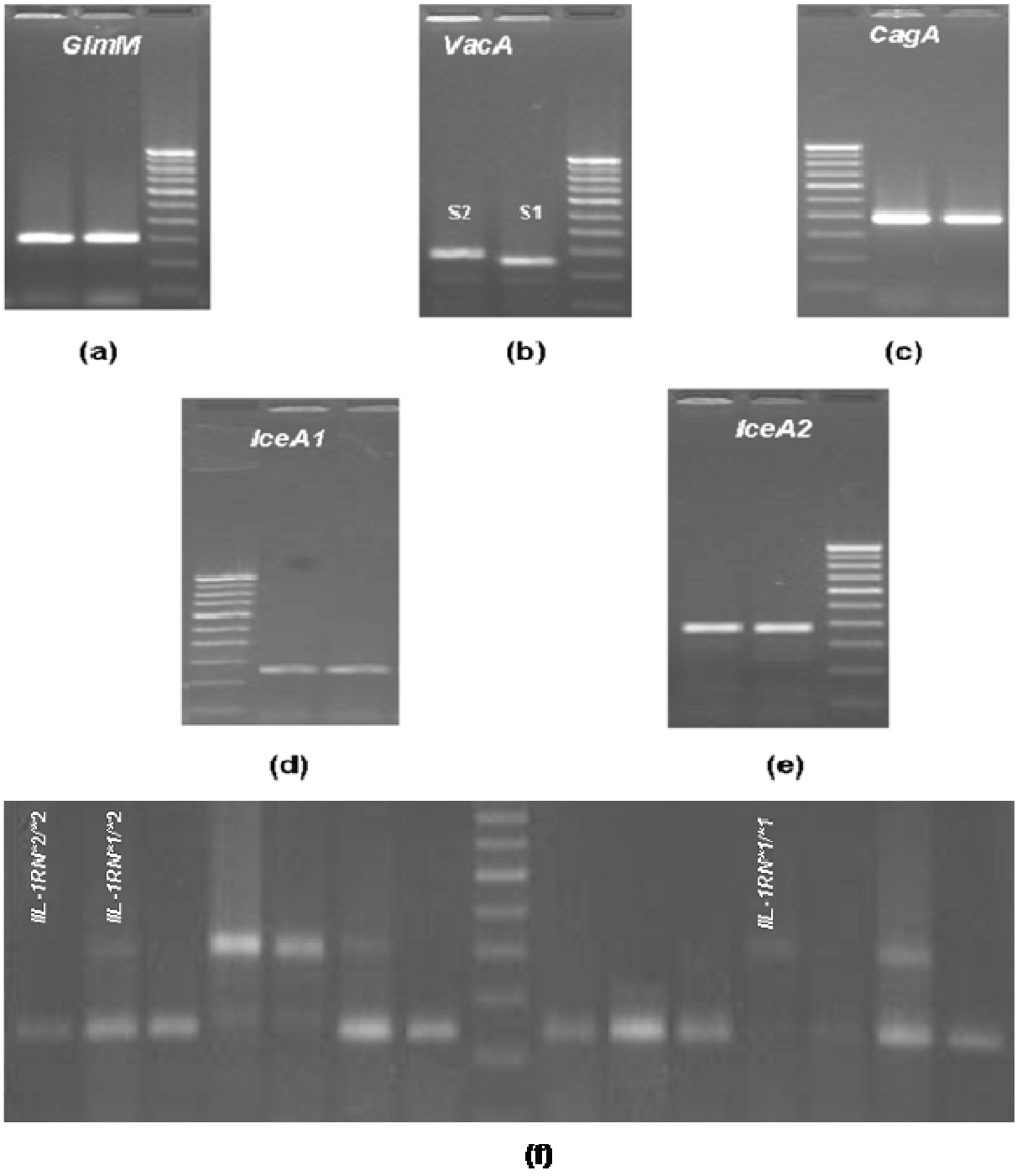

Figure 1. PCR amplification of *H. pylori* genes *glmM* **(a)** *vacA* s region **(b)** *cagA* **(c)** *iceA1* **(d)** *iceA2* **(e)** and host *IL-1RN* gene polymorphism **(f)** from gastric mucosal biopsies collected from dyspepsia patients. The PCR product were electrophoresed on 1.5% agarose gel along with 100 bp DNA ladder and visualized by ethidium bromide staining.

gastric mucosal biopsy from patients previously diagnosed positive for *H. pylori* colonization using RUT and PCR amplification for *glmM* gene (Figure 1a). *vacA* signal region genotyping was done using a primer set differentiating 27 bp and yielding a PCR amplified product of 254 bp and 281 bp for s1 and s2 genotype respectively (Figure 1b). *vacA*s1 genotype was found in 74% (31/42) of patients and one patient colonized with both genotypes. *cagA* status in association with *vacA*s1 genotype has been implicated as a high risk factor for *H. pylori* related gastric pathologies. In this study, we found *cagA* genotype (Figure 1c) in 45% (19/42) of patients

colonized with *H. pylori.* Out of that, 79% (15/19) was presented with *vacA*s1 genotype showing increased risk for developing gastric pathologies among this group of patients.

iceA genotypes of *H. pylori* in colonized dyspepsia patients

i*ceA1* and i*ceA2* are two different genes, which are present at the same genomic locus among various strains of *H. pylori*. i*ceA1* expression is up regulated by contact with epithelium and in some populations this phenotype is associated with peptic ulcer. In this study, we have analyzed the i*ceA1* and i*ceA2* status along with v*acA*s, c*agA* and *IL-1RN* status among patients (Figure 1d and e). ***IceA1*** genotype alone were colonized in 81% of the patients and 19% colonized with *iceA2* genotype only. Interestingly, out of the total samples studied, 31% infected with both *iceA1* and *iceA2* genotypes of *H. pylori.*

IL-1 Receptor antagonist gene polymorphism in *H. pylori* colonized dyspepsia patients

*IL-1RN*2/*2* allele was reported to be associated with pro-inflammatory changes in different pathological conditions. In this study we have analysed the *IL-1RN* allele polymorphism among a group of patients who have colonized with *H. pylori*. *IL-1RN*2/*2* allele were found in 43% (18/42), *IL-1RN*1/*1* allele in 21% (9/42) and heterozygous allele *IL-1RN*1/*2* in 36% (15/42) of patients analysed by PCR amplification using specific primers for VNTR region of the intron 2 which yielded 240 bp (two repeats) and 412 bp (four repeats) product for *IL-1RN*2/*2* and *IL-1RN*1/*1* allele respectively (Figure 1f).

DISCUSSION

H. pylori infection is found to be associated with gastritis, gastric ulcer, MALT lymphoma and gastric cancer in various studies conducted in different parts of the world. Certain virulence genes such as *vacA, cagA,* and *iceA* have significance in pathological changes associated with *H. pylori* infection (Suerbaum and Michetti, 2002; Kusters et al., 2006; Matysiak-Budnik and Mégraud, 2006). Gastric mucosa is well protected against bacterial infections but *H. pylori* is highly adapted to this ecological niche, with a unique array of virulence factors that permit attachment to epithelial cells, evasion of the immune response and persistent colonization. Among a large proportion of infected patients *H. pylori* is not causing any severe pathology for the whole period and it is not clear how they are wading the effect of bacterial virulence factors. In addition to bacterial virulence factors, host genetic system, environment and diet have also been implicated for this discrepancy (Macarthur et al., 2004; Matysiak-Budnik and Mégraud, 2006).

In this study we have used mucosal biopsy from dyspepsia patients as the DNA source for studying the genotype of both bacteria and host. This method will eliminate the chance for pre-selection of single colonies from culture that causes reporting of altered prevalence rate of genotypes among infected patients and reveal the possibility of multi strain infection of *H. pylori* in the gastric epithelium. The presence of *H. pylori* colonization among patients who have tested positive for rapid urease test under routine endoscopic examination was further confirmed by PCR amplification for *glmM* gene. 42 *H pylori* positive samples diagnosed by above method were further analyzed for genotying of *vacA*, *cagA* and *iceA1* and *iceA2*. *vacA*s region was very important in determining the vacoulating action of vacA protein of *H pylori*. *vacA*s2 is found to be less toxic than *vacA*s1, having an additional 27 bp nucleotide sequence probably affecting the hydrophobic characteristic of mature protein and its secretion. We found 74% of patients in this study are infected with *vacA*s1 genotype and associated with 3 peptic ulcer diseases and 39 non-ulcer dyspepsias (NUDs), in which one sample is infected with both *vacA*s1 and s2 genotypes. But there is no significant change was observed in this patient to discuss the importance of this co-existence as reported in an earlier study (Rahman et al., 2003). *cagA* gene is a marker of *cag* Pathogenecity Island found in certain *H. pylori* strains and a crucial virulence factor associated with peptic ulcer diseases and gastric cancer in various studies (Van Doorn et al., 1998; Rugge et al., 1999; Miehlke et al., 2000). We have also analyzed the *cagA* genotype among patients infected with *H. pylori* along with *vacA* signal region genotyping. A strong correlation has been reported for *vacA* s1 and *cagA* genotype of *H. pylori* in gastric carcinoma among patients in Germany (Miehlke et al., 2000). *vacA*s1/*cagA* genotype is more virulent due to the synergistic effect of these toxic proteins on the host system. Among the patients, 45% found to be infected with *cagA* genotype, of which 79% presented with *vacA*s1 genotype and that making those patients at more risk.

Among a short population of patients pathological changes are not severe even in the presence of more virulent bacterial strains. Host genetic background is implicated under these circumstances as a putative factor on determining the severity of diseases. *IL-1 RN* is one of the genes reported to be associated with inflammatory conditions due to its polymorphic region within the second intron containing 2–6 tandem repeats of an 86 bp sequence. In which, the *IL-1RN*2/*2* genotype has been associated with proinflammatory responses more severe and more prolonged than those of other *IL-1RN* genotypes (Hurme and Helminen, 1998). Here we have analyzed the *IL-1RN* gene polymorphism using specific primers differentiating the alleles among patients. The pro-inflammatory *IL-1RN*2/*2* allele was present in 43%

Table 2. *IceA* genotype pattern in association with *vacA* s1, *cagA* and *IL-1RN* allele in *H. pylori* colonized patients.

***H. pylori vacA* s1, *cagA* genotypes & Host *IL-1 RN* polymorphism pattern**	**Number of patients (%)**	***iceA1***	***iceA2***	***iceA1* & *iceA2***
***vacA* s1 group**				
***vacA* s1-*cagA-IL-1RN**2/*2**	**6 (14.3)**	4	-	2
vacA s1-*IL-1RN**2/*2	8 (19.0)	6	1	1
vacA s1-*cagA-IL-1RN**1/*2	7 (16.6)	4	2	1
vacA s1-*IL-1RN**1/*2	6 (14.3)	1	1	4
vacA s1-*cagA-IL-1RN**1/*1	3 (7.1)	2	1	-
vacA s1-*IL-1RN**1/*1	3 (7.1)	2	1	-
Total	**33 (79.0)**	**19**	**6**	**8**
***vacA* s2 group**				
vacA s2-*cagA-IL-1RN**2/*2	-	-	-	-
vacA s2-*IL-1RN**2/*2	4 (9.5)	2	1	1
vacA s2-*cagA-IL-1RN**1/*2	2 (4.8)	1	-	1
vacA s2-*IL-1RN**1/*2	-	-	-	-
vacA s2-*cagA-IL-1RN**1/*1	2 (4.8)	1	-	1
vacA s2-*IL-1RN**1/*1	1 (2.4)	-	1	-
Total	**9 (21.0)**	**4**	**2**	**3**

of patients studied, while the intermediary heterozygous allele *IL-1RN*1/*2* in 36% of patients. *IL-1RN*1/*1* allele was present in the remaining 21% of patients. Other alleles of this gene were not present among this group of patients studied. *vacA* s1/*cagA*/ *IL-1RN*2/*2*-allele were present in 14% of patients studied and *vacA*s1/*cagA*/ *IL-1RN*1/*1* allele in 7% patients, while *vacA*s1/*cagA/IL-1RN*1/*2* allele in 16% of patients. The host pro-inflammatory factors along with bacterial virulence factors may increase the risk for pathological changes leading to gastric cancer among this group of patients. The highest prevalence of gastric abnormalities were reported in patients with both host and bacterial high-risk genotype *vacA*s1/*cagA*/*IL-1RN*2/*2/IL-1B511T* (Rad et al., 2003).

iceA was identified following transcriptional up regulation on contact with gastric epithelial cells (Peek et al., 1998). *iceA* exists as two distinct genotypes, *iceA1* and *iceA2*, and only *iceA1* mRNA is induced following adherence in vitro. *H. pylori iceA1* demonstrates strong homology to a restriction endonuclease *nla*IIR in *Neisseria lactamica* (Xu et al., 2002), and in vivo carriage of *H. pylori iceA1* strains has been reported to be associated with peptic ulceration and enhanced acute neutrophilic infiltration. It has been reported that *iceA1* genotype is predominant in the East Asia, while *iceA2* genotype is predominant in the USA and Columbia (Yamaoka *et al.,*1999). In this study, we have examined the *iceA1* and *iceA2* genotypes along with the high-risk host and bacterial factors. The protein sequences of *iceA1* and *iceA2* reveals patterns of repeated protein cassettes. *iceA1* strains have been reported to be associated with peptic ulceration and enhanced acute neutrophilic infiltration, while *iceA2* strains are more prevalent among patients with asymptomatic gastritis and non-ulcer dyspepsia. In our data, 81% showing *iceA1* genotype and 19% infected with *iceA2* genotype only. Among this 31% patients infected with both strains. *iceA1* and *iceA2* strains were present in all the three peptic ulcer diseases included in this study. It has been reported that the *vacA*s1b, m1 and *iceA1* were closely linked to gastric cancer and 40% of these patients had infected with *iceA1 and iceA2* strains, while a higher prevalence of gastric cancer patients infected with *iceA1/vacA*s1 genotype in South Africa (Kidd et al., 2001). The presentation pattern of *iceA, cagA, vacA* and *IL-RN* genotypes among dyspeptic patients is shown in the Table 2. In conclusion, this study emphasize the need for more population based analysis of the high risk bacterial and host pathogenic factors such as *vacA*s1*/cagA/IL-1RN*2/*2/iceA1/iceA2* combination among *H. pylori* infected patients for better management of treatment strategies.

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