

GENOTYPES OF *HELICOBACTER PYLORI* IN SYMPTOMATIC PATIENTS OF KARACHI, PAKISTAN

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ABSTRACT

Helicobacter pylori infections and gastroduodenal diseases have been reported several times in this region but there is no data regarding prevalence of *H. pylori* *VacA* and *CagA* genotypes in this part of the world. The objective of this study was to determine the prevalence of *H. pylori* genotypes in different gastroduodenal disease patients of Karachi.

Multiple biopsy samples were taken from 227 patients of peptic ulcer disease (PUD), chronic gastritis (CG) and/or abdominal discomfort (AD) in the gastroenterology departments from three different health institutions of Karachi and processed for rapid urease test, culture and histopathology. Positive isolates were further confirmed by PCR amplification of the phosphoglucosamine mutase gene (*glmM*) and analyzed for the occurrence of *CagA* and *VacA* genotypes by PCR.

A significant association between *H. pylori* infection and gastroduodenal diseases was observed ($p < 0.001$). Out of 227 biopsy samples 120 (52.8%) were confirmed positive for *H. pylori* in which 56 were that of PUD, 37 with CG, 19 with AD and 8 with gastric cancer (GCa). Overall 92% isolates from patients with PUD, CG, AD and GCa were *CagA* positive. Eighty five (70.8%) of 120 strains had *VacA* signal sequence genotype s1a, 19 (16%) had type s1b and 16 (13%) had type s2. The *VacA* middle region type m1 and m2 were detected in 78(65%) and 42 (35%) strains. The combinations s1a/m1 and s1a/m2 were found in 66(55%) and 19 (15.8%) respectively. The s1b/m1 and s1b/m2 were found in 12(10%) and 8(6.6%) strains respectively. The s2m2 combinations were identified in 15(12.5%) of 120 isolates. The s2m1 combination was not found in any strain. Forty eight (85.7%) of 56 patients with PUD, 25 (67.5%) of 37 patients with CG, 5 (26.3%) of 19 patients with AD and 7 (87.5%) of 8 with GCa harbored type s1a strains. Genotypes s1a/m1 with positive *CagA* are commonly associated with PUD, CG, AD and GCa.

Key-words: *Helicobacter pylori*, genotype, gastroduodenal disease, Karachi, Pakistan

INTRODUCTION

In developing countries, 70 to 90% of the population carries *H. pylori* and almost all of them acquire the infection before the age of 10 years (Taylor and Parsonnet., 1995). *H. pylori* colonize the gastric mucosa of humans and persist over decades if not treated. It causes chronic infection leading to gastritis. The infection is mostly asymptomatic, but a few individuals will develop severe diseases such as peptic ulcer disease (PUD), gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma (Bickley *et al.*, 1993; Marshall and Warren., 1984).

Several studies have been attempted to identify virulence markers in *H. pylori* allowing the disease outcome of an infection to be predicted, most of them were based on the analysis of *VacA* (vacuolating cytotoxin gene) and *CagA* (cytotoxin-associated gene) status (Van Doorn *et al.*, 1998; Graham and Yamaoka., 1998; Yamaoka *et al.*, 1999; Rudi *et al.*, 2000; Atherton., 2000; Zheng *et al.*, 2000).

The *CagA* gene is one of the 31 genes of a pathogenicity island called the *cag* pathogenicity island (*Cag* PAI) of about 40-kb DNA (Censini *et al.*, 1996; Akopyants *et al.*, 1998). The presence of *CagA* has been considered as a marker of the *Cag* PAI and has been associated with the more virulent *H. pylori* strains (Covacci *et al.*, 1993). About 60% of *H. pylori* isolates possess the *CagA* gene, and nearly all of these express the *CagA* gene product (Covacci *et al.*, 1993; Tummuru *et al.*, 1993). The presence of the *CagA* gene in *H. pylori* strains increases the risk of developing peptic ulceration (Covacci *et al.*, 1993) atrophic gastritis (Tummuru *et al.*, 1993) and adenocarcinoma of the stomach (Blaser *et al.*, 1995; Parsonnet *et al.*, 1997). The *CagA* gene was successfully identified in patients with chronic gastritis and peptic ulcer disease (Tokumaru *et al.*, 1999; Russo *et al.*, 1999) as well as in patients with antral erosions and endoscopic features of normal mucosa (Russo *et al.*, 1999). The *CagA* gene has also been detected in fecal samples of *H. pylori* infected, asymptomatic adult patients (Russo *et al.*, 1999) and of children (Sicinchi *et al.*, 2003).

Virulence factors also include the vacuolating cytotoxin (*VacA*). The *VacA* gene, which is present in all *H. pylori* strains, encodes a cytotoxin that damages epithelial cells by inducing the formation of vacuoles (Cover, TL., 1996) Within the *VacA* gene two variable segments have been identified the signal or s region and the middle or m region (Atherton *et al.*, 1995). The signal sequence exists as s1 or s2 types and type s1 can be subtyped as s1a, s1b

and s1c (Yamaoka *et al.*, 1999; Van Doorn *et al.*, 1998). The middle region occurs as m1 or m2, and among type m1 strains, subtypes m1a and m1b can be distinguished (Atherton *et al.*, 1995; Mukhopadhyay *et al.*, 2000). It has been shown that the presence of *CagA* is strongly associated with *VacA* s1 genotype (Van Doorn *et al.*, 1998). Whereas some studies have suggested that *VacA* s1 genotype, *CagA* and *iceA1* are associated with the development of peptic ulcer disease (Van Doorn *et al.*, 1998; Censini *et al.*, 1996; Atherton *et al.*, 1995), others have failed to confirm the association (Yamaoka *et al.*, 1999; Graham *et al.*, 1996). The discrepant results may be largely due to the fact that there are significant geographical differences in the genotypes among strains.

In the present study, *H. pylori* infection and prevalence of *VacA* and *CagA* genotypes among patient samples from three different health institutions of Karachi was determined and analyzed.

MATERIALS AND METHODS

Patients:

Two hundred and twenty seven patients under going upper gastro duodenal endoscopy for diagnosis and treatment purpose in the gastroenterology departments of three different health institutions of Karachi *viz.*, Liaquat National Hospital (LNH), Pakistan Medical Research council (PMRC) and Jinnah Post Graduate Medical Centre (JPMC-Ward 7) were included in this study. The Institutional Review Board of KIRAN (Karachi Institute of Radiotherapy and Nuclear Medicine) Hospital and Pakistan Medical Research council (PMRC) approved the research protocol. All patients gave written consent for biopsy sampling.

Endoscopy, Biopsy sampling and Histopathology:

Four gastric biopsy specimens from antrum and corpus from each patient were collected for culture, histopathology and PCR. Biopsy specimens for culture and PCR were taken into screw capped bottle containing 0.9% saline and transported in the Microbiology and molecular biology Lab at KIRAN Hospital Karachi. The fourth biopsy specimen was directly used for genotyping by PCR. Biopsies were processed as previously described (Hanif *et al.*, 2009).

Rapid Urease Test: One biopsy specimen from each sample was introduced with a sterile medium in to a semisolid 2% urea agar and incubated at room temperature. Results were recorded up to 4 h after inoculation (Deltenre *et al.*, 1989).

Culture: Biopsies were processed as previously described (Hanif *et al.*, 2009).

DNA extraction from biopsy specimens

Biopsies for DNA extractions were processed as previously described (Hanif *et al.*, 2009).

DNA extraction from *H. pylori* cultures

Genomic DNA was extracted from each strain of *H. pylori* using commercially available kit (Gentra, PUREGENE USA) according to the manufacturer's instructions and dissolved in distilled water and processed as previously described (Hanif *et al.*, 2009).

H. pylori diagnostic PCR

The presences of *H. pylori* DNA was confirmed by PCR amplification of the phosphoglucosamine mutase gene, *glmM* and were processed as previously described (Hanif *et al.*, 2009).

PCR amplification of *CagA*

PCR amplification of *CagA* was carried out using two primer sets named *CagA1/CagA2* and *CagA5/CagA2* (Pan *et al.*, 1997). Primer sequences and their locations in the gene are shown in Table 1. 10 ng of DNA was used in a standard PCR mixture of 25 μ l containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 3.0 mM MgCl₂, and 0.1 mg of bovine serum albumin per ml. The incubation conditions with primer set 1 were as follows: 40 cycles of 1 min at 95°C, 1 min at 55°C, and 1.5 min at 72°C and final 5-min incubation at 72°C. When primer set 2 was used the number of cycles was 35 and the annealing temperature was 60°C. PCR products were analyzed by horizontal agarose (1.5%) gel electrophoresis with ethidium bromide staining. Negative and positive control amplifications were performed in every experiment.

Table 1. Primer sequences, their position in the gene and expected product sizes.

<u>Primer sequences for CagA1/CagA2 and CagA5/CagA2</u>		
<i>CagA1</i>	(5'-GATATAGCCACTACC ACCAC CG-3')	(1249 to 1270) (570 bp)
<i>CagA2</i>	(5'-GGAAATCTTTAATCTCAGTT CGG-3')	(1797 to 1819)
<i>CagA5</i>	(5'-GGCAATGGTG GTCCTGGAGCTAGGC-3')	(1495 to 1519) (324 bp)
<i>CagA2</i>	(5'-GGAAA TCTTT AATCT CAGTT CGG-3')	(1797 to 1819)
<u>Primer sequences for VacA Signal sequence variants</u>		
s1a	5' GTCAGCATCACACCGCAAC 3' 5' CTGCTTGAATGCGCCAAAC 3'	(866–1055) (190 bp)
s1b	5' AGCGCCATACCGCAAGAG 3' 5'CTGCTTGAATGCGCCAAAC 3'	(869–1055) (187 bp)
s2	5' GCTTAACACGCCAAATGATCC 3' 5' CTGCTTGAATGCGCCAAAC 3'	(371–569) (199 bp)
<u>Primer sequences for vac A middle region variants</u>		
m1	5'GGTCAAAATGCGGTCATGG 3' 5' CCATTGGTACCTGTAGAAAC 3'	(2741–3030) (290 bp)
m2	5' GGAGCCCCAGGAAACATTG 3' 5' CATAACTAGCGCCTTGAC 3'	(976–1327) (352 bp)

Table 2. Distribution of *H. pylori* positive samples in various gastroduodenal diseases.

	Liaquat National Hospital (LNH)		Pakistan Medical Research council (PMRC)		Jinnah Post Graduate Medical Center (JPMC)	
	No. of samples collected	H.pylori Positive	No. of samples collected	H.pylori Positive	No. of samples collected	H.pylori Positive
Total number of samples	85	55 (64.7%)	81	43 (53%)	61	22 (36%)
Peptic Ulcer Disease (PUD)	35	25(71.4%)	40	23(57.5%)	26	8 (30.7%)
Chronic gastritis (CG)	30	21(70%)	20	8 (40%)	21	8(40%)
Abdominal discomfort (AD)	20	9(45%)	10	6 (60%)	10	4 (40%)
Gastric Carcinoma (Ca)	0	0	11	6(54.5%)	4	2 (50%)

PCR amplification of VacA

The oligonucleotides forward and reverse primers were synthesized from IDT, USA are listed in Table 1. PCR was performed in 50 µl reaction mixtures containing 100 ng of genomic DNA, 250 nM of each primer, 1X reaction buffer, 1.5 mM MgCl₂, 1 U of *Taq-DNA polymerase* and distilled water in a PCR system (Thermo Hybaid, USA). After denaturation at 94°C for 5 min, amplification was carried out for 27 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s; the mixture was then cycled at 72°C for 7 min to complete the elongation step and was finally stored at 4°C (Atherton *et al.*, 1995). For identification of the amplified products, 10 µl of the PCR mixture was analyzed

by electrophoresis on 2% agarose gel (Serva, USA), stained with ethidium bromide 0.5 mg/ml and read under UV illumination.

Table 4. Number of patients, type of disease, numbers of *H. pylori* positive samples and *VacA* genes in various gastroduodenal diseases.

	Total	Peptic ulcer Disease (PUD)	Chronic gastritis (CG)	Abdominal discomfort (AD)	Gastric cancer (GCa)
No. of patients	227	101(44.4%)	71(31.2%)	40(17.6%)	15(6%)
<i>H. pylori</i> Positive	120	56(46.6%)	37(30.8%)	19(15.8%)	8(6.6%)
<i>CagA</i> Positive	109	52(47.7%)	34(31.1%)	15(13.7%)	8(7.3%)
<i>VacA</i> signal type					
<i>VacA s1a</i>	85	48(56.4%)	25(29.4%)	5(5.8%)	7(8.2%)
<i>VacA s1b</i>	19	6(31.5%)	9(47.3%)	3(15.7%)	1(5.2%)
<i>VacA s2</i>	16	2(12.5%)	3(18.7%)	11(68.7%)	0(0%)
<i>VacA</i> middle region					
<i>VacA m1</i>	78	46(58.9%)	24(30.7%)	0(0%)	8(10.2%)
<i>VacA m2</i>	42	11(26.1%)	14(33.3%)	17(40.4%)	0(0%)

Table 4. Six possible combinations of *VacA* homologue containing signal sequence and middle region of *VacA* alleles and their relationship with *CagA* in various gastroduodenal diseases.

	Peptic ulcer Disease (PUD)		Chronic gastritis (CG)		Abdominal discomfort (AD)		Gastric cancer (GCa)	
		<i>CagA</i> +		<i>CagA</i> +		<i>CagA</i> +		<i>CagA</i> +
Total	56(46.6%)	52	37(30.8%)	34	19(15.8%)	15	8(6.6%)	8
<i>VacA s1a/m1</i>	36(54.5%)	34	20(30.3%)	19	4(6%)	3	6(9%)	6
<i>VacA s1a/m2</i>	12(63%)	11	5(26.3%)	4	1(5.2%)	1	1(5.2%)	1
<i>VacA s1b/m1</i>	4(33.3%)	3	5(41.6%)	4	2(16.6%)	1	1(8.3%)	1
<i>VacA s1b/m2</i>	2(25%)	2	4(50%)	4	1(12.5%)	1	0	0
<i>VacA s2/m1</i>	0	0	0	0	0	0	0	0
<i>VacA s2/m2</i>	2(13.3%)	2	3(20%)	3	10(66.6%)	8	0	0

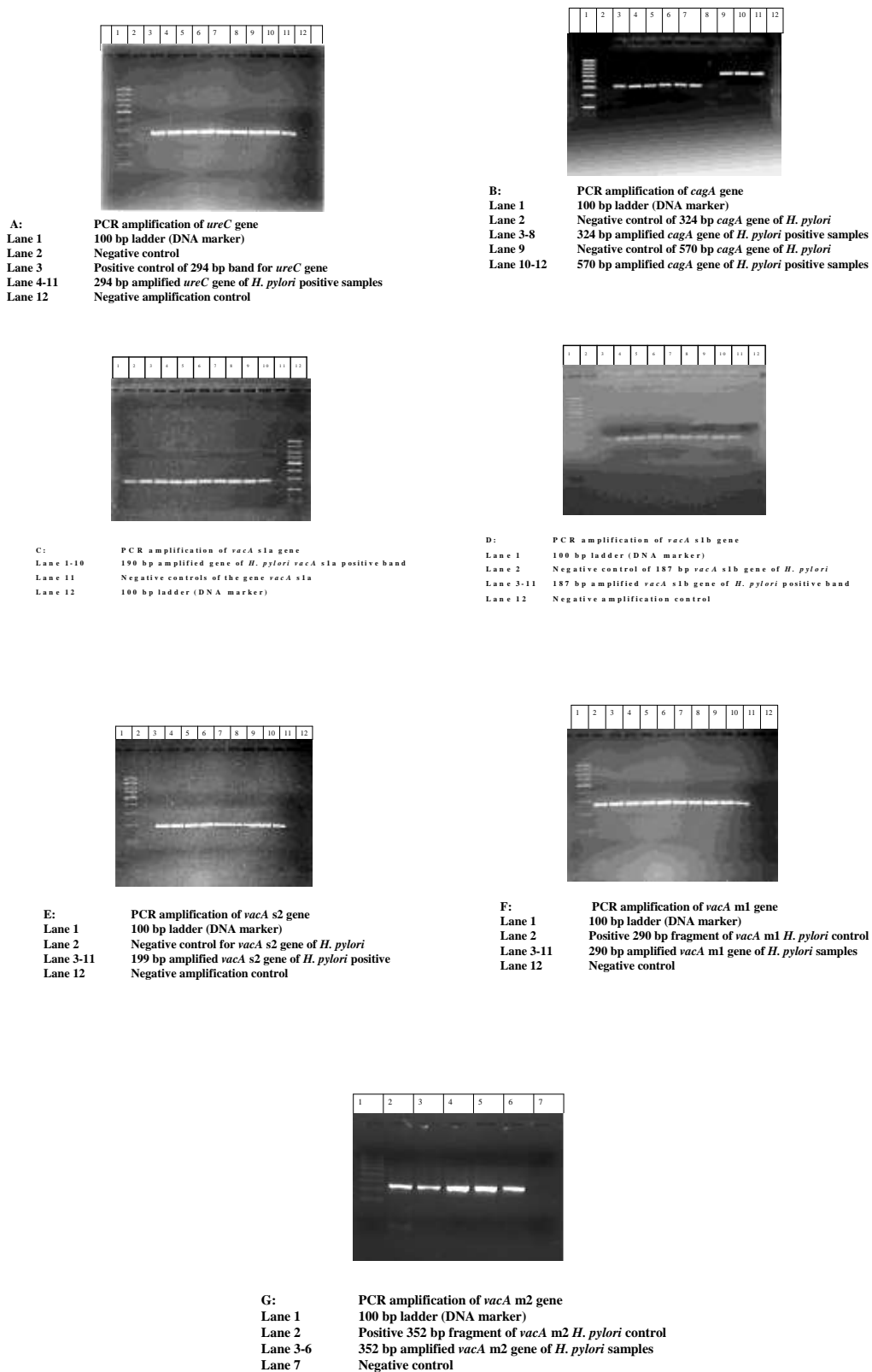


Fig 1. Agarose 1.5% gel electrophoresis of PCR products for *H. pylori* genotyping of representative strains. A. *ureC* gene; B. *Cag A* gene; C. *VacA s1A* gene; D. *VacA s1B*; E. *VacA s2*; F. *VacA m1* and G. *VacA m2* gene subtypes.

RESULTS

Patients consisted of 113 females and 114 males (Mean age 44 years; age range 18 to 78 years). Out of 113 females 58 were *H. pylori* positive and out of 114 males 62 were *H. pylori* positive. A total of 227 biopsy samples were collected and patient's history record revealed peptic ulcer in 101 patients, chronic gastritis in 71 patients, and abdominal discomfort in 40 patients and gastric carcinoma in 15 patients (Table 2). Only 120 (52.8%) were confirmed *H. pylori* positive by PCR (band size of 294 bp) (fig. 1a) and other methods. Among 120 infected patients 56 were that of peptic ulcer disease (PUD), 37 with chronic gastritis (CG), 19 with abdominal discomfort (AD) and 8 with gastric cancer (GCa).

Prevalence of *CagA* gene

A total of 109 patient samples out of 120 (90.8%) were found *CagA* positive, out of them 52 (92.8%) patients having PUD, 34 (91.8%) have CG, 15 (78.9%) have AD and 8 (100%) have GCa (Table 2). The *CagA* gene was detected using specific set of *CagA1/CagA2* primer having 98% sensitivity (band size of 324bp) and primer set *CagA5/CagA2* having 100% sensitivity (band size of 570 bp) (Fig. 1b).

Prevalence of *VacA* gene

The *VacA* gene was detected in all 120 *H. pylori* isolates using primers s1a, s1b, s2, m1 and m2 (Table 1). Eighty-five (70.8%) of 120 strains yielded 190 bp product representing *VacA* signal sequence genotype s1a, 19 (16%) yielded the 187 bp product representing genotype s1b and 16 (13%) yielded 199 bp product representing genotype s2 (Figure 1c, 1d, 1e).

DNA sequences of all 120 *H. pylori* strains amplified either by the primers m1 representing type m1 (290 bp), or by the primers m2 specific for type m2 (352 bp) (Table 1, Fig 1f). Seventy eight (65%) isolates were classified as type m1 and forty two (35%) were classified as type m2 (Table3).

Association of particular *VacA* genotypes with the occurrence of different gastrointestinal diseases was also determined. Forty eight (85.7%) of 56 patients with PUD, 25 (67.5%) of 37 patients with CG, 5 (26.3%) of 19 patients with AD and 7 (87.5%) of 8 with GCa harbored type s1a strains. Type s1b strains were found in only 6 (10.7%) of 56 patients with PUD compared with 9 (24.3%) of 37 subjects with CG, 3(15.7%) of 19 with AD and 1(14.2%) of 8 with GCa. Similarly only two strains were isolated from patients with PUD type s2; three strains were isolated from patients with CG type s2 and 11(57.8%) of 19 with AD, while no strain was isolated with type s2 in patients of GCa (Table 3).

Five of six possible combinations of *VacA* homologue containing signal sequence and middle region (s1a/m1, s1a/m2, s1b/m1, s1b/m2, and s2/m2) were estimated. The s1a/m1 and s1a/m2 combinations were found in 66(55%) and 19 (15.8%) respectively. The s1b/m1 and s1b/m2 were found in 12(10%) and 8(6.6%) strains respectively. The s2m2 combinations were identified in 15(12.5%) of 120 isolates. The s2m1 combination was not found in any strain (Table 4).

Distribution of combinations of *VacA* homologue containing signal sequence and middle region (s1a/m1, s1a/m2, s1b/m1, s1b/m2, and s2/m2) with the occurrence of different gastrointestinal diseases was also determined (Table 4). The s1a/m1 was predominant combination found in 36(64%) of 56 patients with PUD, 20(54%) of 37 patients with CG, 4(21%) with AD and 7(85.7%) with GCa respectively (Table 4).

Association of *VacA* genotypes with *CagA* gene

When the *VacA* signal sequence type was compared with *CagA* status, 79 (92.9%) of 85 *VacA* type s1a strains, 16 (84.2%) of 19 *VacA* type s1b strains and 14 (87.5%) of 16 *VacA* type s2 strains were *CagA* positive (Table 4). This result suggests the close association between *CagA* status and the s1a signal sequence.

A significant association was also found between *VacA* mid-region typing and *CagA* status. Among the 78 m1-type strains 71 (91%) were positive for *CagA* gene and 38 (90.4%) of 42 *VacA* type m2 strains were *CagA* positive.

DISCUSSION

In this study, we have investigated dyspeptic patients having complaints of peptic ulcer, chronic gastritis and/or abdominal discomfort in the gastroenterology departments from three different health institutions of Karachi by endoscopy and gastric biopsies for *H. pylori* infection and their molecular characterization. The present study confirmed the high prevalence of *H. pylori* in symptomatic patients of Karachi previously reported by other authors as well (Hanif *et al.*, 2009; Abbas *et al.*, 1998; Kazi *et al.*, 1990). We found that *ureC* PCR was at least as sensitive as culture for detecting *H. pylori* infection. The rapid-urease test and histological examinations of biopsy specimens

detected *H. pylori* infection in slightly fewer patients than did culture but they still yielded good specificity. Overall, our results are in good agreement with other data for comparisons of different routine tests for *H. pylori* in which Giemsa staining of histological sections (Deltenre *et al.*, 1989) or the agreement among all the tests was taken as the gold standard (Fabre *et al.*, 1989). Overall, PCR assays detected *H. pylori* infection in a higher percentage of patients.

In present study *H. pylori* isolates were characterized on the basis of *VacA* (vacuolating cytotoxin gene) and *CagA* (cytotoxin-associated gene). Overall 90 % isolates were found to have *CagA* gene (*CagA* +) in which 92% have peptic ulcer disease, 91% have gastritis, 79% have abdominal discomfort and 100% have GI cancer carried the *CagA* gene. A high percentage of infections with *CagA* positive isolates were similarly reported from India, China, Japan, Korea, and Singapore (Yamaoka *et al.*, 1999; Zheng *et al.*, 2000; Mukhopadhyay *et al.*, 2000; Pan *et al.*, 1997; Hua *et al.*, 2000). Conversely, reports from Germany, Netherlands and Italy indicated lower overall infection with *CagA* positive isolates and suggested that gastric colonization by such isolates was associated with a higher risk of ulcer development (Van Doorn *et al.*, 1998; Rudi *et al.*, 2000; Carratolil *et al.*, 2000). These inconsistencies between reports imply that *CagA* may or may not be a universal virulence marker.

The *VacA* signal sequence genotypes (s1a, s1b, and s2) were identified in all *H. pylori* strains. For determination of the *VacA* mid-region genotypes, oligonucleotide primers m1 and m2 was allowed to characterize 100% of the *H. pylori* strains isolated from patients of three different health institutions of Karachi. *H. pylori* strains with the *VacA* signal sequence type s1a were predominant, whereas strains with type s1b and s2 rarely occurred, although in contrast with reports on *H. pylori* isolates from other countries (Van Doorn *et al.*, 1998; Yamaoka *et al.*, 1999). The equally important finding complements the strong association between peptic ulcer disease and *VacA* type s1a strains that *VacA* type s2 strains are rarely associated with peptic ulceration.

The *VacA* genotypes of our *H. pylori* isolates were identical to those reported from India (Mukhopadhyay *et al.*, 2000). However, in India, 7.2% strains had mixed s1 and s2 genotypes. Multiple *VacA* s genotypes, frequently reported in other countries (Van Doorn *et al.*, 1998), were totally absent in our study.

We found that the s1a/m1 family of *VacA* alleles of *H. pylori* is predominant in this study, which differ from the findings from other Asian countries (Sicinchi *et al.*, 2003) where the genotypes s1a/m2 are more common. The *VacA* genotype s2/m1 was not identified in our study, which is in consistent with the pioneering study of Atherton, and co-workers (Atherton *et al.*, 1995) who also failed to detect the s2/m1 genotype, suggesting that strains with this genotype suffer from a selective disadvantage or are not viable. However, *VacA* signal sequence type s1, particularly s1a, and genotype s1a/m1 appeared to occur more frequently in our study.

A strong genetic association between of *CagA* and *VacA* signal sequence type s1a were found in this study, a significant association was also found between *VacA* mid-region typing and *CagA* status. Why two genetic elements without any physical linkage on the *H. pylori* chromosome should be so closely associated, is not clear. One hypothesis is that there are two clonal *H. pylori* populations (*VacA* s1/*CagA* and *VacA* s2/*CagA*). Another possibility is that there may be a functional linkage, whereby a selective advantage conferred by each gene product is manifested only in the presence of the other.

It is clear that *H. pylori* strains with the *VacA* signal sequence type s1a and middle region allele m1 were predominant in all the samples collected from three institutions of Karachi. All combinations of these *VacA* alleles occurred, with the exception of s2/m1. Type s1a strains were associated with ulcer and the presence of the *CagA* gene. The findings suggest that *CagA* + *H. pylori* strains with the *VacA* genotype s1a increase the risk for peptic ulcer diseases. Thus, *VacA* genotyping may allow identification of infected subjects at different risk levels.

ACKNOWLEDGEMENTS

We are thankful to the following physicians and paramedical staff for their support and cooperation during the collection of biopsy samples and providing data of the patients investigated. Dr. Sarwar Jehan Zuberi, Dr. Huma Qureshi and Dr. waqar Ahmed (PMRC, Karachi). Dr. S.M Munir, Dr. Azhar, Dr. Sohail Marfani (JPMC, Ward-7, Karachi). Dr. Arfain, Mr. Tabraiz (Liaqat National Hospital, Karachi).

We are also thankful to the following for their help and support during this work. Miss Dilshad Fatima (KIRAN, Karachi). Mr. Tanveer and Mr. Mushtaq (KIBGE, Karachi).

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(Accepted for publication June 2009)