Original Article

Prevalence, diversity and disease association of Helicobacter pylori in dyspeptic patients from Pakistan

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Abstract

Introduction: The etiological association of *Helicobacter pylori* with gastric ulcer (GU), gastric cancer (GC), and duodenal ulcer (DU) is well-known. Understanding the epidemiology of *H. pylori* facilitates the estimation of disease burden in a certain population. This study presents the diversity of *H. pylori* genotypes and their association with different clinical outcomes among dyspeptic patients in Pakistan over a period of four years.

Methodology: Gastric biopsy samples from a total of 450 dyspeptic individualswere subjected to PCR, genotypingand histology.

Results: A total of 201 (45%) cases were found positive for *H. pylori.* The detection rate was high in GU (91%), DU (86%) and GC (83%) cases compared with those cases who had intact gastric mucosa (18%). Histology revealed the presence of infection in 68% of cases of mild/chronic nonspecific gastritis with others belonging to the GU sequel. *cagA* gene carriage was observed in 104 (51%) cases or mostly from DU, GU and GC groups, of which 97 were Western type strains while 3 were East-Asian type strains that are rarely observed in South Asia. *vacA* allelic variant s1am1 was most commonly observed, followed by s1am2, and s1bm1, with direct correlation in diseased cases (gastritis, GU, DU and GC). Prevalent genotypic combinations were s1am1/*cagA*$^-$ in gastritis and s1am1/*cagA*$^+$ in DU, GU, and GC.

Conclusions: Our study indicates the predominant circulation of Western type *cagA* and *vacA*s1am1 type *H. pylori* strains in Pakistan.

Key words: *H. pylori*; *cagA*; *vacA*; duodenal ulcer; gastric ulcer; Pakistan

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Introduction

Helicobacter pylori are associated with various gastrointestinal disorders such as gastritis, gastric ulcer (GU), and duodenal ulcer (DU) [1,2]. In last two decades, it has been associated with gastric adenocarcinoma (GC) [3-7], and it is now defined a class I carcinogen by the World Health Organization (WHO).

H. pylori carries various virulence factors, such as vacuolating cytotoxin A (VacA), CagA cytotoxin and sialic acid binding adhesin (SabA), that are implicated in the development of pathological conditions of gastric mucosa [9]. CagA is encoded by the cytotoxin associated gene A (*cagA*) present in the *Cag* pathogenicity island [10,11]. *H. pylori* strains can be grouped as Western and East Asian subtypes on the basis of polymorphism in the 3′ repeat region of the *cagA* gene [12]. Variable numbers of repeat sequences in each subtype vary the size of protein product and subsequently affect the pathogenicity of *cagA*$^+$ *H. pylori* strains. The East Asian subtype of the *cagA* gene is more commonly associated with severe histological damage, *e.g.*, acute gastritis and gastric cancer, than Western subtypes [13-16]. Each subtype is prevalent in its respective geographical location; *i.e.*, East Asian type *cagA* is prevalent in East Asia and Western types are prevalent in North America and Europe [17]. Thus prevalence is considered both as an effective assessment tool to analyze the epidemiology

of the region and also as a marker to track human migration.

The VacA toxin encoded by the *vacA* gene is another virulence factor of *H. pylori* that induces cytoplasmic vacuoles formation and mitochondrial damage to gastric epithelial cells [18]. Pleomorphic combinations of signal (s) and middle (m) regions of the *vacA* gene affect the amount of vacuolating activity. Due to variable toxin activity, different *vacA* genotypes have been associated with different histological conditions [11] and are considered molecular markers of disease progression. Among all genotypic combinations, s1am1 and s1bm1 are considered to be the most virulent and are frequently reported in patients with acute gastritis [19], peptic ulcer [20], and GC [21], followed by s1m2,which is responsible for moderate toxin activity mainly due to the binding capacity of the m2 region with restricted cell types [22]. Although s2m1 and s2m2 are rare (especially s2m1) and are considered the least toxic of the genotypic combinations because of their inability to form vacuoles, they have been reported in patients with DU and GU [20,23].

Pakistan is considered among the countries with a high rate of gastroduodenal pathologies, especially DU; however, unlike the East Asian rim, the frequency of GC is low [24]. Several studies reported the high rate of *H. pylori* infection in the country based on serological, bacteriological, and histological observations [25-27]. Recent reports, however, document the prevalence of the *vacA* s1m1 genotype in Pakistan, endorsing the common trend of other South Asian neighbors [28,29]. The presence of the organism in drinking water has also been reported, indicating greater chances of infection for the community [30,31]. This study presents a four-year long assessment of *H. pylori* infection, prevalence of *cagA* and *vacA* genotypes, and their relatedness with clinical disease outcome among high-risk patients in Karachi, Pakistan.

Methodology

Patients

A total of 450 dyspeptic patients who underwent gastroduodenal endoscopy at a tertiary care hospital in Karachi were enrolled for the study. Recruitment was conducted from March 2005 to November 2008. Formal written consent was taken from every patient at the time of sample collection. Only patients with no previous therapy with anti-*Helicobacter* (antibiotic and proton pump inhibitor) and anti-cancer drugs and no history of immediate international migration were included in the study. A questionnaire-based approach was used to generate background clinical and social information, while personal interviews were conducted with those patients who had difficulties completing the questionnaire. Patients were grouped on the basis of the gastric endoscopic appearance as "normal" in cases of intact mucosa, gastric ulcer (GU), duodenal ulcer (DU), gastritis, and gastric cancer (GC). The study was conducted withapproval from the ethical review board, University of Karachi, Pakistan.

Sample collection and processing

Three gastric biopsy specimens (two from antrum and one from fundus) were collected from each patient and kept in sterile 20% glucose solution [32] for DNA extraction and in 10% formaldehyde (Scharlau, Barcelona, Spain) for histology. Samples were immediately transported to the laboratory in a light-proof insulated ice box and processed within an hour. After homogenization in sterile water, DNA was extracted using the SDS-PK method as described previously [33]. Samples were stored at -20^{o}C. PCR analysis for the human β-globulin housekeeping gene was performed to check the quality of DNA.

Histology

Histology was conducted on 166 samples from patients who had damaged gastric mucosa and who were positive for *H. pylori* by PCR. Samples from normal (n = 26) and GC cases (n = 6) were not included because of endoscopically declared intact mucosa and extremely diverse gastric pathology respectively. The 3 µm paraffin-embedded sections of biopsy samples were cut and stained with hematoxylin and eosin (H and E) for histological examination. The Sydney system was applied for histological slide examination [34]. Scoring for gastritis and gastritis activity, from mild to severe, was based on the level of infiltrating lymphocytes and neutrophils, respectively. Grading in bacterial density was performed by observing the bacterial count present on gastric epithelia. The presence of large bacterial clumps was considered as severe, whereas a single or small group of two to three organisms was graded from mild to moderate. Other important parameters including atrophy, intestinal metaplasia, and chronic or active gastritis, were also considered important.

Detection of H. pylori

PCR detection of *H. pylori* was performed using specific primers targeting 16S rRNA and *ureA* genes (Table 1). For PCR amplification, 800 ng of DNA

Table 1. List of primers used for molecular characterization of *H. pylori*

		Primer	Sequence	Product size
***H. pylori* identification**	16S rRNA	HP1	CTGGAGAGACTAAGCCCTCC	109 bp
	UreA	HP2 HP64-F HP64-b	ATTACTGACGCTGATTGTGC TCACCCCAAAAGAGTTAGAC GAAGTGTGAACCGATTTGAA	428bp
cagA		CAGT-F	ACCCTAGTCGGTAATGGG	variable
		CAGT-R	GCTTTAGCTTCTGAYACYGC	
		cagA-F	TTGACCAACAACCACAAACCGAAG	183bp
		cagA-R	CTTCCCTTAATTGCGAGATTCC	
	Western type	CAGT-F	ACCCTAGTCGGTAATGGG	variable
		CAGW-R	TGCCCTACAMCACCSAAACCAC	
		CAGW-F	AAAAATTGACCRACTCAATC	variable
		CAGT-R	GCTTTAGCTTCTGAYACYGC	
	East Asian type	CAGT-F	ACCCTAGTCGGTAATGGG	variable
		CAGJ-R	GCAATTTTGTTAATCCGGTC	
		CAGJ-F	GCATCAGCAGGTAAAGGAGT	variable
		CAGT-R	GCTTTAGCTTCTGAYACYGC	
vacA	s1 and s2	VA1-F	ATGGAAATACAACAAACACAC	s1=259bp
		VA1-R	CTGCTTGAATGCGCCAAAC	s2=286bp
	m1and m2	VAG-F	CAATCTGTCCAATCAAGCGAG	m1=645bp
		VAG-R	GCGTCAAAATAATTCCAAGG	m2=570bp
	s1a	SS1-F	GTCAGCATCACACCGCAAC	190bp
	s1b	SS3-F	AGCGCCATACCGCAAGAG	187bp
	S1c	SIC-F	CTCGCTTTAGTGGGGCTA	213 bp
		VA1-R	CTGCTTGAATGCGCCAAAC	

samples were added to a PCR mixture containing 0.5 mM forward and reverse primers, 1.5 mM $MgCl_2$, 1U of Taq polymerase (Invitrogen, Milan, Italy), 2.5 µl PCR buffer (Qiagen, Germany), and 200 µM of dNTPs. PCR amplifications were performed according to previously described protocols [35,36].

cagA *and* vacA *genotyping*

cagA status was determined for *H. pylori* positive samples by polymerase chain reaction (PCR) using two different primers pairs as described previously [12,37] (Table 1). The *cagA* gene was typed using two different sets of primers (Table 1) targeting the specific first repeat (FR) and Western second repeat regions (WSR) for the Western type, and the specific FR and East Asian second repeat regions (ESR) for the East Asian type as previously described [12,38]. The total number of repeats present in each region was determined according to amplicon size.

vacA genotyping (s1, s2, m1, m2) was performed as previously described [39,40]. Subtypes were differentiated by amplicon size (Table 1). The s1 region was subtyped by PCR reactions using specific primers as previously described by Atherton *et al.* [40].

Statistical analyses

Descriptive analysis was performed on a total of 201 *H. pylori* positive samples using Statistical Package for Social Science version 17 (SPSS IBM, Chicago, USA). Pearson Chi-square test was applied to analyze the significant distribution of various genotypes in different groups of patients. Association trends were determined by Goodman and Kruskal's lambda (λ) test by cross-tabulation.

Results

A total of 450 dyspeptic patients, 274 males and 176 females ranging in age from 10 to 80 years (average age of 38 ± 15.8), were included in the study. Forty-five (10%) patients had previous family history of gastric ulcer and/or gastritis. The clinical symptoms ordinarily found included dyspepsia, malaise, vomiting, stomachache, and epigastrium pain.

Clinical diagnosis along with gastric endoscopic observations identified 305 (67%) cases with different gastroduodenal lesions, whereas 145 (32%) samples from patients who had intact gastric mucosa were considered normal. Among 305 cases, 217 (71%) were diagnosed as gastritis, 45 (14.7%) as duodenal ulcer, and 36 (11.8%) as gastric ulcer, while 6 (2%) samples showed gastric cancer, later confirmed by histology.

Of the 450 samples analyzed, 201 (45%) were positive for *H. pylori* by PCR assays targeting the *ureA* and 16SrRNA genes; these included 45% (98/217) of the identified gastritis cases, 91% (41/45) of the DU cases, 86% (31/36) of the GU cases, and 83% (5/6) of the GC cases. Furthermore, 26 (18%) of the normal group patients were found positive for *H. pylori* (Table 2).

Table 2. Distribution of *cagA* and *vacA*genotypes

Description	Total		Normal		Gastritis		Gastric Ulcer		Duodenal Ulcer		Gastric Cancer	
	N	%	N	%	N	%	N	%	N	%	N	%
Total HP positive	201		26		98		31		41		5	
cagA positive	104	51	3	11	48	49***	23	74***	26	63** *	4	80* **
• *Western type cagA*	97		3		46		23		25		4	
• *East Asian type cagA*	3		0		2		0		1		0	
vacAs1am1	94	47	9	35	42	43*	22	71*	19	46	3	60*
vacA s1am2	40	20	6	23	24	24	1	3	9	22	1	20
vacAs1bm1	11	5	0	0	4	4	3	10	4	10	0	0
vacAs1bm2	13	6	2	8	6	6	2	6	2	5	1	20
vacAs1cm2	4	2	0	0	1	1	1	3	2	5	0	0
vacAs2m1	28	14	9	35**	17	17	0	0	2	5	0	0

*** P <0.0005, ** P < 0.005, * P < 0.05

H. pylori *with different histopathological findings*

Since histological changes predict the clinical outcome of disease in the future, association of *H. pylori* with histopathology is important. In this study we applied the Sydney scoring system to quantify the level of bacterial density, gastritis, and gastritis activity to bioptic samples. Histopathology of the samples positive for *H. pylori* revealed that out of 166 samples, 57 had mild and chronic non-specific gastritis while 24 samples exhibited severe gastritis patterns. No specific grade of gastritis was found associated with bacterial density. Other important pathological trends including follicular gastritis with mild to moderate glandular activity (n = 12), mild and chronic gastritis with intestinal metaplasia (n = 17), atrophic gastritis (n = 18), non-atrophic gastritis (n = 22), and chronic gastritis with focal activity (n = 16) were also noticed in *H. pylori* positive samples.

cagA *and* vacA *genotyping*

In this study the prevalence of *cagA* gene was 52% (104/201), of which 97 were Western types and 3 were East Asian types (Table 2). Four *cagA* positive samples were not successfully typed.As shown in Table 2, the presence of *cagA* gene was observed in 49% (48/98) of the patients with gastritis, 63% (26/41) with DU, 74% (23/31) with GU, and 80% (4/5) with GC (P <0.005), whereas 11% (3/26) of the normal cases were also positive. East Asian type strains were observed in three samples, two of gastritis and one of DU cases.

The *vacA* genotyping revealed the presence of six different allelic variants of the *vacA* gene in our samples. As shown in Table 2, s1am1 genotype was predominantly detected in 47% (94/201) of *H. pylori* positive cases, followed by s1am2, s2m1, s1bm2, s1bm1, and s1cm2. Mixed *vacA* genotypes were observed in 11 cases, indicating the presence of more than one *H. pylori* strain for each case. Detailed analysis revealed that 22 (71%) of the GU and 3 (60%) of the GC cases had s1am1 genotype (P < 0.0005), which was significantly higher than what was seen in the DU and normal cases. Respective percentages of s1am2 were 24% and 22%, s1bm1 4% and 10%, s1bm2 6% and 5%, and s2m1 17% and 5% in patients with gastritis and DU. Three (60%) GC patients carried s1am1, 1(20%) s1am2 and 1(20%) s1bm2 (Table 2).

Association and clinical correlation of virulence genes combinations

Table 3 shows that 54 (57%) *H. pylori* strains had the *vacA* s1am1/*cagA*$^+$ combination, while 40 (42%) were *vacA*s1am1/*cagA*$^-$, indicating that the s1am1 is present in our population irrespective of *cagA* gene expression ($P \geq 0.05$). However, *vacA*s1am2 was more significantly associated with *cagA*$^+$ (26; 65%) than *cagA*$^-$ cases (14; 35%) (P ≤ 0.005). In contrast, the s2m1 genotype was more commonly found in *cagA*$^-$ strains (21; 75%) than in *cagA*$^+$ (7; 25%) (P ≤ 0.0005).No s1cm2 genotypes were observed in *cagA*$^+$ cases.

Distribution analysis further showed that 17% of the gastritis patients had s1am2/*cagA*$^+$ and 16% had s1am1/*cagA*$^+$, followed by the other combinations. Interestingly, 68% of the GU and 32% of the DU patients also exhibited the s1am1/*cagA*$^+$ combination, followed by the other combinations, as listed in Table 3. Out of five GC cases, two (40%) carried the s1am1/*cagA*$^+$ combination while s1am1/*cagA*$^-$, s1am2/*cagA*$^+$ and s1bm2/*cagA*$^+$ were also observed. Interestingly, a number of patients with normal gastric mucosa carried s2m1/*cagA*$^-$; however, association of s2m1 with the *cagA* gene was seen in 3% and 5% of the cases with gastritis and DU respectively (P < 0.05) (Table3).

Table 3. Disease association of *cagA*and *vacA*gene combinations

Description	Total		Normal		Gastritis		Gastric Ulcer		Duodenal Ulcer		Gastric Cancer	
	N	%	N	%	N	%	N	%	N	%	N	%
Total HP positive	201		26		98		31		41		5	
Western type cagA/vacAs1am1	54	57	2	3	16	16	21	68	13	32	2	40
Western type cagA/vacAs1am2	26	13**	1	4	17	17	0	0	7	17	1	20
Western type cagA/vacAs1bm1	8	72	0	0	3	3	2	6	3	7	0	0
Western type cagA/vacAs1bm2	1	8	0	0	0	0	0	0	0	0	1	20
Western type cagA/vacAs2m1			0	0	3	3	0	0	2	5	0	0

Discussion

Etiological association of *H. pylori* with gastric ulcer, duodenal ulcer, and gastric adenocarcinoma is well-known. Although the organism is believed to colonize half of the world's population [41], its association with clinical outcomes differs widely. Moreover, its genetic diversity is largely structured in various ethnic groups and geographical locations. In Asia, the prevalence of *H. pylori* infection differs widely in different countries; for example, Pacific Asians such as Chinese, Korean and Japanese populations are considered to be highly susceptible groups due to the highestprevalence of *H. pylori* infection and GC cases in those countries compared to rest of the world. Comparatively, other countries of the region such as Singapore, Malaysia, Taiwan and Vietnam are considered to be at intermediate risk due to lower prevalence *H. pylori* and GC. On the other hand, epidemiology of *H. pylori* in South Asia presents a very distinct view with a low number of GC cases and high rate of *H. pylori* infection, presumably due to the genetic diversity of the pathogen [42]. In spite of the geographic proximity of these countries, differences in prevalence among different ethnic groups and geographic regions have been reported.

In this study, we observed the presence of *H. pylori* infection in 45% of dyspeptic cases of patients who resided in the southern province of Sindh, Pakistan, mainly in Karachi. In 2004, other authors reported a prevalence of *H. pylori* infection in 56% of dyspeptic patients from Karachi [43]; however, in early 2000, Taj *et al.* reported seroprevalence of *H. pylori* in 80% of the cases from the city [27]. Our report and another from Yakoob *et al.* confirm the decreasing trend of *H. pylori* infection overtime in Karachi, which is comparable to trends seen in other South Asian and European countries [44-46]. Interestingly, earlier studies from northern and central parts of Pakistan reported 66% and 84% prevalence rates among dyspeptic patients [25,29], indicating the difference in *H. pylori* infection in geographic niches and ethnic groups in Pakistan. Interestingly *cagA* gene analysis further confirms the above-mentioned observation. We found that 52% of *H. pylori* strains carried the *cagA* gene with the positivity rate of 80% in GC, 74% in GU, 63% DU, and 11% in normal cases, which is comparable with the observations of a previous report from Karachi, Pakistan [47], but conflicts with a different previous study conducted in the northern and central parts of Pakistan which reported a 26% *cagA* positivity rate. A literature search focusing on studies performed in close geographic proximities revealed that overall *cagA* postivity ratios are still lower in Pakistan compared to those in neighbouring countires. Previous studies reported results similar to ours or a little higher, with percentages in Iran and Afghanistan at 60% and 67%, respectively [48]; however, our typical South Asian neighbours, such as India and Bangladesh, reported much higher rates [49]. Previously we have also identified that antibiotic resistance in *H. pylori*, which corelates with the absence of the *cagA* gene, is more prevalent in Pakistan compared to the prevalence seen in neigboring countries [50], indicating the difference in epidemiological characteristics of this region.The difference in *H. pylori* infection rates and in *cagA* positivity among various Pakistani cities can be related to either the circulation of different strains or to the susceptibility to *H. pylori* infection of the different ethnicities living in Pakistan. Nonetheless, the analysis of only the 3′end of the *cagA* gene might be considered as a major limitation to the study, and it is important to perform PCR for the *cagA* empty site and dot hybridization. It would be worthwhile to study the genetic makeup and ancestry of *H. pylori* strains circulating in these regions.

In this study we observed the pathogenic attributes of *H. pylori.* As expected, *H. pylori* infection was more highly associated with patients with damaged gastric mucosa, such as gastric ulcer (86%) andduodenal ulcer (91%), than with dyspeptic patients with intact gastric mucosa (18%). This observation contrasts earlier studies which report the approximately equal rate of *H. pylori* infection in patients with normal and damaged gastric mucosa [28]. Histological examination of *H. pylori* infected cases further showed the presence of gastric ulcer promoting conditions such as follicular, mild and chronic gastritis with intestinal metaplasia and atrophic gastritis. Apart from the stronger association with diseased cases, *H. pylori* infection was observed only in 45% of the gastritis patients. Given that *H. pylori* was not involved in the induction of gastritis in rest of the patients, it is difficult to predict the other causes. Previous reports suggest that non-*H. pylori* gastritis is primarily present in pediatric patients or others with underlying co-morbidities such as inflammatory bowel disease, autoimmune, and genetic diseases [51]. However, our studied population mainly constituted of adult patients with no known co-morbidities; therefore, it may be plausible that other factors such as fungal and viral infections, excessive intake of NSAIDs or other drugs, and immunological disorders are responsible of inducing gastritis in these

patients, as previously observed in other countries [52].

Understanding the epidemiology of *H. pylori* infection has been greatly facilitated by molecular typing methods which reveal genetic variations in the *vacA* gene and allow the estimation of future clinical outcomes. Low infection rates coupled with stronger clinical associations, high *cagA* positivity, and the predictive change in the gastritis pattern prompted us to scrutinize these samples for various *vacA* genotypes which are directly linked with the virulence stature of *H. pylori*. Previous studies have suggested that *vacA* genotypes s1a and s1b are prominently associated with high toxin activity. Our study on *vacA* genotyping, as expected, revealed significant association of these subtypes, *i.e.*, s1am1, s1bm1 with diseased cases such as gastritis, gastric ulcer and duodenal ulcer when compared with patients who had intact gastric mucosa; however, the situation was more prominent in gastritis cases. Published reports from different geographical regions provide this evidence despite other differences in *H. pylori* genotypes [6], but contrary to their observations, we did not find the s1cm1 type in our studied population.

Another striking feature of our study was the presence of the s2m1 *vacA* genotype in certain gastritis and normal cases. Although s2m1 positive *H. pylori* strains have previously been observed in many countries including Cuba, India, China, South Africa and the Middle East [53-56], they are still regarded as an "alien" in gastroenterology due to their infrequent presence. Such strains are generally considered to be non-toxic [57], which, to some extent justifies their presence in patients with intact mucosa and indicates less likely involvement in disease pathology. However, it is noteworthy that in our study, s2m1 subtypes were also found in gastritis patients, which indicates the diverse epidemiology of gastritis in the Pakistani population. To monitor whether s2m1 positive samples provide some trend, descriptive analysis was performed with respect to various demographical characteristics including age, gender, and the time of sample collection, but no significant association was observed (data not shown). Since these findings are based on PCR methods, it is important to sequence such strains to come to a final conclusion. Our observations, however, warrant in-depth analysis for s2m1 positive *H. pylori* strains in Pakistan and other neighboring countries.

In conclusion, this investigation presents the distribution pattern of the *cagA* and *vacA* genes of *H. pylori* and their association with gastroduodenal pathologies which could contribute to understanding the trend of *H. pylori* infection in Pakistan.

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References

1. Marshall BJ (1986) Campylobacter pyloridis and gastritis. J Infect Dis 153: 650-657.
2. Marshall BJ, Warren JR (1984) Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1: 1311-1315.
3. Kuipers EJ, Sipponen P (2006) *Helicobacter pylori* eradication for the prevention of gastric cancer. Helicobacter 11: 52-57.
4. Plummer M, Vivas J, Fauchère JL (2000) *Helicobacter pylori* and stomach cancer: a case-control study in Venezuela. Cancer Epidemiol Biomarkers Pre 9: 961.
5. Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ (2001) *Helicobacter pylori* infection and the development of gastric cancer. New Engl J Med 345: 784-789.
6. Wong BCY, Lam SK, Wong WM, Chen JS, Zheng TT, Feng RE, Lai KC, Hu WHC, Yuen ST, Leung SY, Fong DY, Ho J, Ching CK, Chen JS, China Gastric Cancer Study Group (2004) *Helicobacter pylori* eradication to prevent gastric cancer in a high-risk region of China: a randomized controlled trial. JAMA 291: 187-194.
7. Mbulaiteye SM, Hisada M, El-Omar EM (2009) *Helicobacter pylori* associated global gastric cancer burden. FrontBiosci 14: 1490-1504.
8. Ladeira MSP, Bueno RCA, dos Santos BF, Pinto CLS, Prado RP, Silveira MG, Rodrigues MAM, Bartchewsky W, Pedrazzoli J, Ribeiro ML (2008) Relationship among oxidative DNA damage, gastric mucosal density and the relevance of *cagA, vacA* and *iceA* genotypes of *Helicobacter pylori*. Dig Dis Sci 53: 248-255.
9. Yamaoka Y (2008) Increasing evidence of the role of *Helicobacter pylori* SabA in the pathogenesis of gastroduodenal disease. J Infect Dev Cntries 2: 174-181.
10. Mattar R, Marques SB, Monteiro MS, dos Santos AF, Iriya K, Carrilho FJ (2007)*Helicobacter pyloricag* pathogenicity island genes: clinical relevance for peptic ulcer disease development in Brazil. J Med Microbiol 56: 9-14.
11. Basso D, Zambon CF, Letley DP, Stranges A, Marchet A, Rhead JL, Schiavon S, Guariso G, Ceroti M, Nitti D (2008) Clinical relevance of *Helicobacter pyloricagA* and *vacA* gene polymorphisms. Gastroenterology 135: 91-99.
12. Yamaoka Y, Osato MS, Sepulveda AR, Gutierrez O, Figura N, Kim JG, Kodama T, Kashima K, Graham DY (2000) Molecular epidemiology of *Helicobacter pylori*: separation of H. pylori from East Asian and non-Asian countries. Epidemiol Infect 124: 91-96.

13. Argent RH, Hale JL, El-Omar EM, Atherton JC (2008) Differences in *Helicobacter pylori* CagA tyrosine phosphorylation motif patterns between western and East Asian strains, and influences on interleukin-8 secretion. J Med Microbiol 57: 1062-1067.
14. Yamaoka Y, El-Zimaity HMT, Gutierrez O, Figura N, Kim JK, Kodama T, Kashima K, Graham DY (1999) Relationship between the *cagA* 3'repeat region of *Helicobacter pylori*, gastric histology, and susceptibility to low pH. Gastroenterology 117: 342-349.
15. Sicinschi LA, Correa P, Peek RM, Camargo MC, Piazuelo MB, Romero Gallo J, Hobbs SS, Krishna U, Delgado A, Mera R (2010) CagA C terminal variations in *Helicobacter pylori* strains from Colombian patients with gastric precancerous lesions. Clin MicrobiolInfect 16: 369-378.
16. Sgouras DN, Panayotopoulou EG, Papadakos K, Martinez-Gonzalez B, Roumbani A, Panayiotou J, vanVliet-Constantinidou C, Mentis AF, Roma-Giannikou E (2009) CagA and VacA polymorphisms do not correlate with severity of histopathological lesions in *Helicobacter pylori*-infected Greek children. J Clin Microbiol 47: 2426-2434.
17. Kersulyte D, Mukhopadhyay AK, Velapatino B, Su WW, Pan ZJ, Garcia C, Hernandez V, Valdez Y, Mistry RS, Gilman RH, Yuan Y, Gao H, Alarcón T, López-Brea M, Balakrish Nair G, Chowdhury A, Datta S, Shirai M, Nakazawa T, Ally R, Segal I, Wong BC, Lam SK, Olfat FO, Borén T, Engstrand L, Torres O, Schneider R, Thomas JE, Czinn S, Berg DE (2000) Differences in genotypes of *Helicobacter pylori* from different human populations. J Bacteriol 182: 3210-3218.
18. Wada A, Yamasaki E, Hirayama T (2004) *Helicobacter pylori* vacuolating cytotoxin, VacA, is responsible for gastric ulceration. J Biochem 136: 741-746.
19. Gloria P, Raymundo R, Salvador A, Cristina R, Ausencio C, Erasmo N, Sergio V (2009) Frequency of *vacA, cagA* and *babA2* virulence markers in *Helicobacter pylori* strains isolated from Mexican patients with chronic gastritis. Annals ClinMicrobiol Antimicrob 8: 1-14.
20. Atherton JC, Cao P, Peek RM, Tummuru MKR, Blaser MJ, Cover TL (1995) Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. J Biol Chem 270:17771-17775.
21. López-Vidal Y, Ponce-de-León S, Castillo-Rojas G, Barreto-Zúñiga R, Torre-Delgadillo A (2008) High diversity of *vacA* and *cagAHelicobacter pylori* genotypes in patients with and without gastric cancer. PloS One 3: 3849.
22. Letley DP, Atherton JC (2000) Natural diversity in the N terminus of the mature vacuolating cytotoxin of *Helicobacter pylori* determines cytotoxin activity. J Bacteriol 182:3278-3280.
23. De Francesco V, Margiotta M, Zullo A, Hassan C, Giorgio F, Zotti M, Stoppino G, Bastianelli A, Diterlizzi F, Verderosa G (2009) *Helicobacter pylorivacA* arrangement and related diseases: a retrospective study over a period of 15 years. Dig Dis Sci 54: 97-102.
24. Singh K, Ghoshal UC (2006) Causal role of *Helicobacter pylori* infection in gastric cancer: an Asian enigma. World J Gastroenterol 12: 1346-1351.
25. Qureshi TZ, Bilal R, Saleem K, Zafar S (2008) General prevalence of *Helicobacter pylori* infection in dyspeptic population of Islamabad, Pakistan. Nucleus 45: 157-162.
26. Amjad M, Kazmi SU, Quraishy S (1995) Serum immunoglobulin levels of patients with gastroduodenal pathology and *H. pylori* infections. In Proceedings Dept. Biochem, Uni Karachi 55.
27. Taj Y, Essa F, Kazmi SU, Abdullah E (2003) Sensitivity and specificity of various diagnostic tests in the detection of *Helicobacter pylori*. J Coll Physicians Surg Pak 13: 90-93.
28. Yakoob J, Jafri W, Jafri N, Islam M, Abid S, Hamid S, Shah HA, Shaikh H (2005) Prevalence of non-*Helicobacter pylori* duodenal ulcer in Karachi, Pakistan. World J Gastroenterol 11: 3562-3565.
29. Ahmad T, Sohail K, Rizwan M, Mukhtar M, Bilal R, Khanum A (2009) Prevalence of *Helicobacter pylori* pathogenicity associated *cagA* and *vacA* genotypes among Pakistani dyspeptic patients. FEMS Immunol Med Microbiol 55: 34-38.
30. Rasheed F, Khan A, Kazmi SU (2009) Bacteriological analysis, antimicrobial susceptibility and detection of 16S rRNA gene of *Helicobacter pylori* by PCR in drinking water samples of earthquake affected areas and other parts of Pakistan. Malaysian J Microbiol 5: 123-127.
31. Khan SS, Zulfiqar A, Danish KF, Sauwal M, Bashir S, Zaman S (2008) Prevalence of *Helicobacterpylori* infection in patients with gastroduodenal disease in Pakistan. Rawal Med J 33: 1-6.
32. Covacci A, Censini S, Bugnoli M, Petracca R, Burroni D, Macchia G, Massone A, Papini E, Xiang Z, Figura N (1993) Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc Natl Acad Sci U. S. A. 90: 5791-5795.
33. Khan S, Jaffer NN, Khan MN, Rai MA, Shafiq M, Ali A, Pervez S, Khan N, Aziz A, Ali SH (2007) Human papillomavirus subtype 16 is common in Pakistani women with cervical carcinoma. Int J Infect Dis 11: 313-317.
34. Price AB (1991) The Sydney system: histological division. J Gastroenterol Hepatol 6:209-222.
35. Chisholm SA, Owen RJ, Teare EL, Saverymuttu S (2001) PCR-based diagnosis of *Helicobacter pylori* infection and real-time determination of clarithromycin resistance directly from human gastric biopsy samples. J Clin Microbiol 39: 1217-1220.
36. Miyabayashi H, Furihata K, Shimizu T, Ueno I, Akamatsu T (2000) Influence of oral *Helicobacter pylori* on the success of eradication therapy against gastric *Helicobacter pylori.* Helicobacter 5: 30-37.
37. Sicinschi LA, Correa P, Peek Jr RM, Camargo MC, Delgado A, Piazuelo MB, Romero-Gallo J, Bravo LE, Schneider BG (2008) *Helicobacter pylori* Genotyping and Sequencing Using Paraffin-Embedded Biopsies from Residents of Colombian Areas with Contrasting Gastric Cancer Risks. Helicobacter 13: 135-145.
38. Acosta N, Quiroga A, Delgado P, Bravo MM, Jaramillo C (2010)*Helicobacter pylori* CagA protein polymorphisms and their lack of association with pathogenesis. World J Gastroenterol 16:3936.
39. Chisholm SA, Teare EL, Patel B, Owen RJ (2002) Determination of *Helicobacter pylorivacA* allelic types by single step multiplex PCR. Let App Microbiol 35: 42-46.
40. Atherton JC, Cover TL, Twells RJ, Morales MR, Hawkey CJ, Blaser MJ (1999) Simple and accurate PCR-based system for typing vacuolating cytotoxin alleles of *Helicobacter pylori.* J Clin Microbiol 37: 2979-2982.

41. IARC (1994) Schistosomes, liver flukes and Helicobacter pylori. Lyon, France World Health Organization, International Agency for Research on Cancer.
42. Fock KM and Ang TL (2010) Epidemiology of *Helicobacter pylori* infection and gastric cancer in Asia. J Gastroenterol Hepatol 25: 479-486.
43. Yakoob J, Jafri N, Jafri W, Zaman S, Bian LC, Islam M, Hussainy AS, Zaman V (2004) Polymerase chain reaction in the detection of *Helicobacter pylori* infection. J Coll Physicians Surg Pak 14: 153-156.
44. Katelaris PH, Tippett GH, Norbu P, Lowe DG, Brennan R, Farthing MJ (1992) Dyspepsia, *Helicobacter pylori*, and peptic ulcer in a randomly selected population in India. British Med J 33: 1462-1466.
45. Ghoshal UC, Tiwari S, Dhingra S, Pandey R, Ghoshal U, Tripathi S, Singh H, Gupta VK, Nagpal AK, Naik S (2008) Frequency of *Helicobacter pylori* and CagA antibody in patients with gastric neoplasms and controls: the Indian enigma. Dig Dis Sci 53: 1215-1222.
46. Asfeldt AM, Straume B, Steigen SE, Løchen ML, Florholmen J, Bernersen B, Johnsen R, Paulssen EJ (2008) Changes in the prevalence of dyspepsia and *Helicobacter pylori* infection after 17 years: the Sørreisa gastrointestinal disorder study. Eur J Epidemiol 23: 625-633.
47. Yakoob J, Abid S, Abbas Z, Jafri W, Ahmad Z, Ahmed R, Islam M (2009) Distribution of *Helicobacter pylori* virulence markers in patients with gastroduodenal diseases in Pakistan. BMC Gastroenterol 9: 87.
48. Dabiri H, Bolfion M, Mirsalehian A, Rezadehbashi M, Jafari F, Shokrzadeh L, Sahebekhtiari N, Zojaji H, Yamaoka Y, Mirsattari D (2010) Analysis of *Helicobacter pylori* Genotypes in Afghani and Iranian Isolates. Polish J Microbiol 59: 61-66.
49. Udhayakumar G, Senthilkumar C, Jayanthi V, Devaraj N, Devaraj H (2009) *Helicobacter pylori* detection and genotyping in gastric biopsy specimens from Chennai patients. Canadian JMicrobiol 55: 126-132.
50. Khan A, Farooqui A, Manzoor H, Akhtar SS, Quraishy MS, Kazmi SU (2012) Antibiotic resistance and cagA gene correlation: A looming crisis of *Helicobacter pylori*. World J Gastroenterol 18: 2245-2252.
51. Genta R, Sonnenberg A (2012) Non-*Helicobacter pylori* gastritis is common among paediatric patients with inflammatory bowel disease. Aliment Pharmacol Ther 35: 1310-1316.
52. Lauwers GY, Fujita H, Nagata K, Shimizu M (2010) Pathology of non-*Helicobacter 2 pylori* gastritis: extending the histopathologic horizons. J Gastroenterol 45: 131-145.
53. Torres LE, Melián K, Moreno A, Alonso J, Sabatier CA, Hernández M, Bermúdez L, Rodríguez BL (2009) Prevalence of *vacA*, *cagA* and *babA2* genes in Cuban *Helicobacter pylori* isolates. World J Gastroenterol 15: 204-210.
54. Devi SM, Ahmed I, Francalacci P, Hussain MA, Akhter Y, Alvi A, Sechi LA, Mégraud F, Ahmed N (2007) Ancestral European roots of *Helicobacter pylori* in India. BMC Genomics 8: 184.
55. Sugimoto M, Zali MR, Yamaoka Y (2009) The association of *vacA* genotypes and *Helicobacter pylori*-related gastroduodenal diseases in the Middle East. Eur JClin Microbiol Infect Dis 28: 1227-1236.
56. Tanih NF, McMillan M, Naidoo N, Ndip LM, Weaver LT, Ndip RN (2010) Prevalence of *Helicobacter pylorivacA, cagA* and *iceA* genotypes in South African patients with upper gastrointestinal diseases. Acta Tropica 116: 68-73.
57. Pagliaccia C, de Bernard M, Lupetti P, Ji X, Burroni D, Cover TL, Papini E, Rappuoli R, Telford JL, Reyrat JM (1998) The m2 form of the *Helicobacter pylori* cytotoxin has cell type-specific vacuolating activity. Proc Natl Acad Sci U S A 95: 10212-10217.

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