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Research Paper

Investigation of the association between clinical outcome and the cag pathogenicity-island and other virulence genes of *Helicobacter pylori* isolates from patients with dyspepsia in Eastern Turkey

Gokben Ozbey[1], Ulvi Demirel[2], Cem Aygun[2], Hasan Basri Ertas[3]

[1]Vocational School of Health Services, Firat University, Elazig, Turkey.
[2]Department of Gastroenterology, Faculty of Medicine, Firat University, Elazig, Turkey.
[3]Department of Microbiology, Faculty of Veterinary Medicine, Firat University, Elazig, Turkey.

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Abstract

The aims of our work were to determine the presence of the *cag* pathogenicity-island (cag PAI) and other virulence genes of *Helicobacter pylori* recovered from patients with gastritis and peptic ulcer, and to investigate the correlation of these virulence genes with clinical outcome. The presence of the *cagA*, the promoter regions of *cagA*, *cagE*, *cagT*, and the left end of *cag*-PAI (LEC), cag right junction (*cag*RJ), the plasticity region open reading frames (ORFs), *vacA* and *oipA* genes among 69 *H. pylori* isolates were determined by polymerase chain reaction. Intact *cag* PAI was detected in only one (1.4%) isolate. The *cagA* gene was identified in 52.1% and 76.2% of isolates from patients with dyspepsia (gastritis and peptic ulcer), respectively. The plasticity region ORFs *i.e.* JHP912 and JHP931 were predominantly detected in isolates from peptic ulcer. Less than 25% of the isolates carried other ORFs. Types I, II and III were the most commonly found among the isolates. None of the isolates possessed type Ib, 1c, IIIb, IV and V motifs. The most commonly *vacA* genotypes were s1am1a and s1m2 in isolates with peptic ulcer and gastritis, respectively. The results confirmed that the prevalence of *oipA* (Hp0638) gene was 75% and 85.7% in patients with gastritis and peptic ulcer, respectively. Furthermore, *vacA* s1am1a positivity was significantly related to peptic ulcer ($p < 0.05$).

Key words: *Helicobacter pylori*, gastritis, peptic ulcer, cag pathogenicity-island, polymerase chain reaction.

Introduction

Helicobacter pylori (*H. pylori*) is a bacterial pathogen which can cause gastritis, peptic ulcer and gastric carcinoma (Cremonini *et al.*, 2001; Saunders *et al.*, 2005). Strains of *H. pylori* are classified into two types (types I and II) (Xiang *et al.*, 1995: Hofman *et al.*, 2000). Type I is a pathogenic form, correlates with severe disease status, expresses functional vacuolating cytotoxin A (*vacA*) and includes an approximately 40 kb cluster located at 3' end of the *cag* pathogenicity island (*cag* PAI) (Censini *et al.*, 1996; Ikenoue *et al.*, 2001; Kersulyte *et al.*, 2000; Mattar *et al.*, 2007). Type II which is less virulent and includes a non-pathogenic form of *vacA*, lacks *cag* PAI (Censini *et al.*, 1996; Backert *et al.*, 2004).

The *cag* PAI is separated the two groups (*cag*I and *cag*II) by a novel insertion sequence called IS*605* and these include at least 14 and 16 open reading frames (ORFs), respectively (Censini *et al.*, 1996; Akopyants *et al.*, 1998; Audibert *et al.*, 2001; Mattar *et al.*, 2007). The cytotoxin associated gene E (*cagE*) gene which is needed for the induction of interleukin (IL)-8 from gastric epithelial cells is located in the *cag*I (Censini *et al.*, 1996; Ikenoue *et al.*, 2001; Tan *et al.*, 2005). The *cagT* gene has been reported to be a marker of the *cag*II region (Mattar *et al.*, 2007) and correlates with severe clinical outcomes (Mattar *et al.*, 2007; Pacheco *et al.*, 2008).

Comparison of the genome sequence analysis of *H. pylori* 26695 and J99 strains demonstrated several regions of different G+C contents (Tomb *et al.*, 1997; Alm *et al.*,

Send correspondence to G. Ozbey. Vocational School of Health Services, Firat University, 23119 Elazig, Turkey. E-mail: gokben.ozbey@yahoo.com.

1999; Occhialini *et al.*, 2000; Salih *et al.*, 2007). From these regions, a large region in strains J99 and 26695 has been named as the "plasticity region" (Alm *et al.*, 1999; Doig *et al.*, 1999; Salih *et al.*, 2007). In the J99 plasticity region (JHP914 to JHP951), the authors reported to observed to be 38 ORFs while 33 ORFs were not included in *H. pylori* 26695, and the majority of the ORFs encode putative proteins with unknown function (Occhialini *et al.*, 2000). However, some of ORFs have been determined to share similarity to genes encoding proteins included in DNA replication (JHP919 and JHP931) and other functions (JHP941 and JHP951) (Occhialini *et al.*, 2000; Salih *et al.*, 2007).

Till date, we studied on the presence of several genes, such as *cagA*, *vacA*, *cagE*, induced by contact with epithelium (*iceA)* and blood adhesion binding antigen (*babA2)* among adults (Ozbey *et al.*, 2012) and children (Ozbey *et al.*, 2013) in Eastern Turkey. However, the data on identification of *cag* PAI and multiple virulence genes of *H. pylori* in Turkey is scarce. This study aimed to identify the presence of *cag* PAI and other virulence genes of *H. pylori* isolates from dyspeptic patients with gastritis and peptic ulcer in Elazig Province, the East of Turkey as well as to evaluate the relevance between the clinical outcome and the *cag* PAI and other virulence genes.

Materials and Methods

Isolates

A total of 69 *H. pylori* isolates (48 cases of gastritis and 21 cases of peptic ulcer) obtained from Turkish dyspeptic patients attending Gastroenterology Unit of Firat University Hospital between May and December 2011 were analyzed for the presence of *cag* PAI and other virulence genes. Ethics approval was given by the Medical Ethics Committee at Firat University and informed consent was ensured from each participant.

DNA extraction and Determination of the *cag*-PAI and other virulence genes

DNA samples from *H. pylori* isolates were extracted by QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's guidelines.

PCR analyses were performed to amplify *cagA*, the *cagA* promoter region, *cagE*, *cagT*, and the LEC of the *cag* PAI, as described elsewhere (Ikenoue *et al.*, 2001; Kauser *et al.*, 2004) (Table 1).

Primers which designed by Kersulyte *et al.* (2000), Mukhopadhyay *et al.* (2000), Veralovic *et al.* (1991) and Kauser *et al.* (2005a) were used to determine the presence of the cag right junction (*cag*RJ), the plasticity region ORFs, *vacA* and *oipA* (Hp0638) genes (Table 1).

Amplification reactions were performed using 2XPCR Master Mix kit (#K01071, Fermentas) following the manufacturer's instructions in touchdown thermal cycler (Hybaid, England) with PCR conditions shown in Table 1. Ten μL aliquot of each amplicon was expose to gel electrophoresis on a 1.5% agarose gel and visualised using a UV transilluminator.

Statistical analysis

Fischer's exact and χ^2 tests were used to analyze significant differences between the *cag* PAI and other virulence genes of *H. pylori* isolates with the clinical outcome. A probability of less than 0.05 was evaluated significant.

Results

Table 2 shows the distribution of the *cag* PAI and other virulence genes of *H. pylori* isolates from cases of gastritis and peptic ulcer. The prevalence of LECI, LECII, *cagE*, the promoter region of the *cagA* and *cagA* were detected more (14.3%, 19%, 38.1%, 47.6% and 76.2%, respectively) in isolates from peptic ulcer. One isolate (1.4%; 1 of 69) were observed to possess the intact *cag* PAI.

Types I (6.3%), II (4.2%) and III (8.3%) were observed predominantly in isolates from gastritis. However, Ia (19%) and IIIa (23.8%) motifs were the most common types in peptic ulcer isolates. None of the isolates contained type Ib, 1c, IIIb, IV and V motifs. The most predominant plasticity region ORFs were JHP912 and JHP931 and these two ORFs were identified more in isolates from peptic ulcer. Less than 25% of the isolates carried other ORFs (JHP926, JHP933, JHP944, JHP945, JHP986). The *vacA* s1am1a was the most extensively *vacA* genotype found in isolates with peptic ulcer while s1m2 was the most predominant genotype in patients with gastritis. However, no *vacA*s1c, *vacA*m1b and *vacA*s2m1 genotypes were demonstrated in the current study. The *oipA* gene was observed in 75% of isolates with gastritis and 85.7% of isolates with peptic ulcer.

Assesing the association between the *cag* PAI and other virulent genes with clinical outcome, *vacA* s1am1a genotype was shown to be statistically significant with peptic ulcer ($p < 0.05$).

Discussion

Since its first identification by Censini *et al.* (1996) in 1996, the *cag* PAI part of the *H. pylori* genome has been widely studied so far (Olbermann *et al.*, 2010; Rizzato *et al.*, 2012).

Conflicting results have been obtained in studies on the prevalence of *cagA* gene in different geographical regions of the world. The prevalence of the *cagA* gene was 60-70% in Western countries (Rudi *et al.*, 1998) but the prevalence in East Asian countries was detected to be found in more than 90% of cases (Maeda *et al.*, 1998; Yamaoka *et al.*, 1999). This study was similar to that reported in Turkey (Salih *et al.*, 2007) and Western countries (Covacc *et al.*, 1999; Arents *et al.*, 2001) where *cagA* gene were observed

Table 1 - Oligonucleotide sequences and cycle conditions used for detecting the *cag* PAI and the other virulence genes of *H. pylori* isolates in the current study.

Genes	Primer	Oligonucleotide sequence (5'-3')	PCR conditions	Size (bp) of PCR product	References
cag PAI					
*cag*A1	cagA-F1	AACAGGACAAGTAGCTAGCC		701	
	cagA-R1	TATTAATGCGTGTGTGGCTG			
*cag*A2	cagA-F2	GATAACAGGCAAGCTTTTGA		349	
	cagA-R2	CTGCAAAAGATTGTTTGGCAGA	94 °C for 5 min (initial denaturation)		
*cag*AP1	cagAP-F1	GTGGGTAAAAATGTGAATCG	90 °C for 30 s; 52 °C for 30 s	730	
	cagA-R2	CTGCAAAAGATTGTTTGGCAGA	70 °C for 1 min (40 cycles)		
*cag*AP2	cagAP-F2	CTACTTGTCCCAACCATTTT	70 °C for 10 min (final extension)	1181	(Ikenoue *et al*., 2001; Kauser *et al*., 2004, 2005a)
	cagA-R2	CTGCAAAAGATTGTTTGGCAGA			
cagE	cagE-F1	GCGATTGTTATTGTGCTTGTAG		329	
	cagE-R1	GAAGTGGTTAAAAAATCAATGCCCC			
cagT	cagT-F1	CCATGTTTATACGCCTGTGT		301	
	cagT-R1	CATCACCACACCCTTTTGAT			
LECI	LEC-F1	ACATTTTGGCTAAATAAACGCTG		384	
	LEC-R1	TCTCCATGTTGCCATTATGCT			
LECII	LEC-F2	ATAGCGTTTTGTGCATAGAA		877	
	LEC-R2	ATCTTTAGTCTCTTTAGCTT			
cag right junction					
	cagF4584 F (1)	GTTAATACAAAAGGTGGTTTCCAAAAATC		1000/800	
	cagR5280 R (3)	GGTTGCACGCATTTTCCCTTAATC			
	cagF4584 F (1)	GTTAATACAAAAGGTGGTTTCCAAAAATC		400	
	miniIS*605* R (8)	CCGCTAAAGACGATTGGGCTT			
	fcn unk F (6)	TGGATTAAATCTTAATGAATTATCG		350	(Kersulyte *et al*., 2000; Kauser *et al*., 2005a)
	cagR5280 R (3)	GGTTGCACGCATTTTCCCTTAATC	94 °C for 30 s, 52 °C for 30 s		
	fcn unk F (6a)	ACTCTATTTTGCTTGCAGTGCTTTTGG	72 °C for 1 min (30 cycles)	350	
	cagR5280 R (3)	GGTTGCACGCATTTTCCCTTAATC			
	cagF4856 F (4)	GCGATGAGAAGAATATCTTTAGCG		350	
	cagR5280 R (3)	GGTTGCACGCATTTTCCCTTAAT			
	IS606-1692 F (5)	CTAACAATTTGCCATTATGCTGT		2000	
	cagR5280 R (3)	GGTTGCACGCATTTTCCCTTAATC			
	cagF4584 F (1)	GTTAATACAAAAGGTGGTTTCCAAAAATC		400	
	Xins.R (7)	CGCTCTCTAATTGTTCTAGGA			
Plasticity region	JHP912 F	CAATAGCCTTGCTCACGCTTC		624	
ORFs	JHP912 R	GTTAAATGGTGAGAGCCTACG			
	JHP926 F	GATGAGCAAATCAATGGCATG		991	
	JHP926 R	ACCTTTCAATACCGCTAGAAG			

Table 1 (cont.)

Genes	Primer	Oligonucleotide sequence (5’-3’)	PCR conditions	Size (bp) of PCR product	References
	JHP931F	GTATTAGCGAAGTGCAATCAC		1.133	
	JHP931R	GCTAATTTGTTTAGGCGTAGC	94 °C for 5 min (initial denaturation		(Mukhopadhyay *et al*., 2000 Kauser *et al*., 2005a)
	JHP933 F	GAGTGAGTTTAAGCGAAC	94 °C for 1 min, 62°C for 1 min	708	
	JHP933 R	CTTGTTGCTCTTGCAAGG	72 °C for 1 min (35 cycles)		
	JHP944 F	CTATGAGTGAAGAATTAACGC	72 °C for 7 min (final extension)	358	
	JHP944 R	CGCTCCATTCCAATATCTTTG			
	JHP945 F	CAATGCGACTAACAGCATAG		1.028	
	JHP945 R	CGCATTTGCTGTCATCTTTG			
	JHP947 F	GATAATCCTACGCAGAACG		611	
	JHP947 R	GCTAAAGTCATTTGGCTGTC			
	JHP986 F	GCATGTCCCAAATCGTAGG		566	
	JHP986 R	TGCATTTCGCATTGGCTCC			
vacA signal and middle regions					
*vacAs*1 or *vacAs*2	VAIF	ATGAAAAAAACCCTTTTAC		259 (s1)	(Carrol *et al*., 2004)
	VAIXR	CGAATTGCAAGTGATGGT		286 (s2)	
*vacAs*1a	SS1-F	GTCAGCATCACACCGCAAC		190	
	VA1-R	CTGCTTGAATGCGCCAAAC			(Atherton *et al*., 1995)
*vacAs*1b	SS3-F	AGCGCCATACCGCAAGAG		187	
	VA1-R	CTGCTTGAATGCGCCAAAC			
*vacAs*1c	S1C-F	CTCTCGCTTTAGTGGGGYT		213	(Yamazaki *et al*., 2005)
	VA1-R	CTGCTTGAATGCGCCAAAC			
*vac*Am1a	VA3-F	GGTCAAAATGCGGTCATGG	94 °C for 30 s; 54 °C for 30 s	300	
	VA3-R	CCATTGGTACCTGTAGAAAC	72 °C for 1 min (30 cycles)		
vacAm1b	VAm-F3	GGCCCCAATGCAGTCATGGAT		300	(Kersulyte *et al*., 2000; Kauser *et al*., 2005a)
	VAm-R3	GCTGTTAGTGCCTAAAGAAGCAT			
*vac*Am2	VA4-F	GGAGCCCCAGGAAACATTG		400	
	VA4-R	CATAACTAGCGCCTTGCAC			
oipA	HP0638-F	GTTTTTGATGCATGGGATTT	94 °C for 1 min; 52 °C 1 min;	401	(Veralovic *et al*., 1991; Kauser *et al*., 2005a)
	HP0638-R	GTGCATCTCTTATGGCTTT	72°C for 1 min (35 cycles)		

Table 2 - Distribution of the *cag* PAI and the other virulence genes of *H. pylori* isolates from cases of gastritis and peptic ulcer.

cag PAI	Gastritis (n = 48) (%)	Peptic ulcer (n = 21) (%)
LEC1	5 (10.4)	3 (14.3)
LEC2	3 (6.3)	4 (19)
cagT	17 (35.4)	7 (33.3)
cagE	16 (33.3)	8 (38.1)
*cag*AP	8 (16.7)	10 (47.6)
cagA	25 (52.1)	16 (76.2)
cagRJ region		
Type I	3 (6.3)	1 (4.8)
Type Ia	0	4 (19)
Type II	2 (4.2)	0
Type III	4 (8.3)	0
Type IIIa	1 (2.1)	5 (23.8)
ORFs		
JHP912	25 (52.1)	14 (66.7)
JHP926	1 (2.1)	0 (0)
JHP931	15 (31.3)	9 (42.9)
JHP933	10 (20.8)	5 (23.8)
JHP944	8 (16.7)	3 (14.3)
JHP945	11 (22.9)	4 (19)
JHP986	6 (12.5)	1 (4.8)
vacA alleles		
*vac*As1a	35 (72.9)	19 (90.5)*
*vac*As1b	2 (4.2)	0 (0)
*vac*As2	11 (22.9)	2 (9.5)
*vac*Am1a	10 (20.8)	15 (71.4)*
*vac*Am2	38 (79.2)	6 (28.6)
oipA	36 (75)	18 (85.7)

*significant $p < 0.05$.

to be higher in peptic ulcer patients compared to gastritis. We confirmed that no relevance between the *cagA* and gastroduodenal disease in the present study which was in accordance with previous studies (Hussein *et al.*, 2008; Baghaei *et al.*, 2009). However, other studies (Gunn *et al.*, 1998; Basso *et al.*, 2008) represented an association.

Previous studies reported that strains which lack the *cagT* gene had a defective 'molecular syringe' (Rohde *et al.*, 2003; Kauser *et al.*, 2005b). We represented that isolates from gastritis and peptic ulcer carried *cagE* and *cagT* with almost similar proportion. In a study performed in England, most of strains obtained from ulcer patients retained the *cagE* and *cagT* (Kauser *et al.*, 2005b). A pevious study has shown that the *cagE* is a better marker of an intact *cag* PAI in Japanese strains (Ikenoue *et al.*, 2001) which is in contrast with our findings. Kauser *et al.* (2004) and Matteo *et al.* (2007) described that a conserved LEC region was rearranged more in strains related to severe pathology worldwide.

The prevalence of the *cag* PAI varies in different geographical regions. There was only one report concerning the distribution of the *cag* PAI and the ORFs of *H. pylori* strains in Turkey (Salih *et al.*, 2007). Previous reports showed that an intact *cag* PAI gene was highly observed in Japanese, Malaysia and Singapore strains, least found in European and African strains, and very poorly found in Peruvian, Indian, Iranian and Turkish strains (Kauser *et al.*, 20004; Baghaei *et al.*, 2009; Salih *et al.*, 2007; Schmidt *et al.*, 2010). Our results also support the findings (Baghaei *et al.*, 2009; Rudi *et al.*, 1998) indicated that an intact *cag* PAI gene was detected to be low prevalence in Iranian and Turkish strains. This could be due to geographical closeness, the similar condition of life and diet in Iran and Turkey (Baghaei *et al.*, 2009). An intact *cag* PAI may be underestimated when a selective primers were used since *cag* PAI was encoded by ~ 40 kb gene (Schmidt *et al.*, 2010).

Five main types (I, II, III, IV and V) were detected at the *cag* RJ region and scientists reported that the three types (I, II and III) were prevalent (Kersulyte *et al.*, 2000). The authors indicated that type IIIa or type I were observed in 28.8% of the motifs in England strains and some of the European strains share similar profiles with the Asian strains (Kauser *et al.*, 2005b) The results of the current study are also supportive of a previous study that Turkish strains showed to be predominant of types I, II and III which were no associated with the severity of the disease (Salih *et al.*, 2007).

Among the plasticity region ORFs, JHP940 and JHP947 have been observed more in strains with gastric cancer (Occhialini *et al.*, 2000). Our data is similar to the previous reports in Costa Rica, Netherlands and Turkey where the prevalence of JHP0945 was almost similar proportions between *H. pylori* isolates obtained from gastritis and peptic ulcer (Occhialini *et al.*, 2000; de Jonge *et al.*, 2004; Salih *et al.*, 2007) but different from a study (Sugimoto *et al.*, 2012) which demonstrated that the prevalence of JHP0945 was found to be higher in isolates with peptic ulcer. We observed that JHP0931 gene was not associated with clinical disease in the present work which was in consistent with a study in Costa Rica (Occhialini *et al.*, 2000). However, Salih *et al.* (2007) found that JHP912 and JHP931 genes was significant association in cases with peptic ulcer in Turkey.

The *H. pylori oipA* which have great antigenic characteristics and increase the serum level of IL-8 besides the clinically important demonstration of peptic ulcer, is an important virulence factor (Yamaoka *et al.*, 2002; Zambon *et al.*, 2002; Kudo *et al.*, 2004). We showed no significant correlation between the *oipA* gene and peptic ulcer, in contrast with a previous study (Salih *et al.*, 2007) performed in Turkey.

In a study carried out in Turkey, the authors detected that the most predominantly genotype among type II isolates was s1/m2, but except for one patient with gastritis and gastric ulcer possessed s1/m2 genotype, all type I isolates had s1/m1 genotype (Nagiyev *et al.*, 2009). This study showed that none of *H. pylori* isolates had *vac*Am1b genotype. Our study is concurrence with previous studies (Blaser *et al.*, 1995; Salih *et al.*, 2007) which reported that s1a/m1a was the most prevalent genotype among isolates with peptic ulcer. In contrast, s1c/m1b and s1a/m1b strains were the predominant genotypes in East Asian countries (Yamazaki *et al.*, 2005). We found that the s1m2 strains were predominantly detected in isolates from gastritis. Our findings were similar to the previous reports in Turkey (Erzin *et al.*, 2006; Nagiyev *et al.*, 2009) where the *vacA*s1a strains showed to be significantly correlated with peptic ulcer.

In conclusion, this study suggests that *cagA*, *oipA*, JHP912, JHP931 and *vacA* s1am1a were the most common genes in isolates with peptic ulcer, and *vac*As1am1a was significantly correlated with peptic ulcer. When considering the worldwide distribution of *H. pylori* as a common pathogen, further larger scale researches are necessary to be conducted in strains obtained from different geographical regions in order to assess the possible role of *cag* PAI and other virulence genes in different clinical outcomes which is correlated with *H. pylori* infections.

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