Genotyping of *vacA* of *Helicobacter pylori* in patients from Baghdad with gastro-duodenal diseases.

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Abstract

The current cross-section study aimed to molecular identification of *H. pylori* directly from gastric tissue biopsies(GTB) and characterize the more frequent *vacA* genotypes between patients with gastric diseases and their association with disease outcome. Gastric tissue biopsies were collected from 69 patients suffering from symptoms of severe gastro-intestinal tract diseases (GIT), attended to under-went gastro-endoscopy and clinical examination at Gastrointestinal-tract and Liver Disease Hospital, Medical City and Al-Imamein Medical City, Baghdad, Iraq. All patients were subject to the tests included in the current study. Direct identification of *H. pylori* from GTB and detection of *vacA* genotypes were done using conventional PCR. The results shown that between 69 gastric patients, 51 (73.9%)were positive for *H. pylori*. The remaining patients 18/69 (26.1%) were considered as negative control for *H. pylori*. Among 51 *H. pylori* positive samples, genotyping of s and m regions of *vacA* revealed that high frequency of *vacA* s1b as detected in 22/51 (43.14%) patients and s1a in 17/51 (33.3%) patients. The *vacA* m2 was detected in 33/51 (64.71%) patients, in compared to *vacA* m1 which was detected in 15/51 (29.41%) patients. The most frequent *vacA* allelic combination in patients with GU was s1b/m1 followed by s1a/m1. In conclusion, there was no statistically significant association between *vacA* genotypes and clinical outcome (*P*>0.05).

Keywords: *Helicobacter pylori*, *Vac*A genotyping, Clinical outcome, Allelic combination.

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Introduction

Helicobacter pylori (*H. pylori*) is a helix shaped, Gram-negative, microaerophilic, flagellated bacteria [1]. It is the most widely recognized human pathogen around the world, infecting about 50% of the worldwide population [1,2]. It was characterized as a Group 1 carcinogen by the World Health Organization and the International Agency for Research [3,4]. *Helicobacter pylori* colonizing human stomach and the main source for acquired infection is contaminated water or food or poorly disinfected endoscopes [4]. Although nearly 50% of the population is infected with *H. pylori* worldwide, the prevalence, incidence, age distribution and sequels of infection are significantly different in developed and developing countries [5,6]. In all parts of the world, *H. pylori* is the strongest known risk factor for gastric cancer. The incidence of gastric cancer varies markedly throughout the world, and it occurs about twice as commonly in males than females [5,6]. Several Iraqi studies have been conducted to estimate the frequency of *H. pylori* infections. One Iraqi study revealed that *H. pylori* prevalence varied in various ages groups [7]. A second Iraqi study was detected the presence of this bacterium in 58 patients with gastritis B and it found that 55.2% of patients gave positive results of culturing on Columbia agar, and 81% of patients gave positive results to serum IgG anti-*H. pylori* antibodies [8]. Another study was detected the prevalence of *H. pylori* among University students in Erbil/Iraq and was showed that 173/311 (55.8%) students were positive for *H. pylori* using antigen cassette test [9].

Genetically, *H. pylori* is more different than most other bacterial species and the genetic differences of a several virulence factors, for example, urease, CagA and VacA, can be utilized as a tool for predicting the risk of developing different diseases [10-12]. Urease catalyzes the hydrolysis of urea into carbon dioxide and ammonia, consider as a fundamental virulence factor, since it permits *H. pylori* survival in the highly acidic and hostile environment of the lumen of the stomach before it reaches the mucus layer [11,12]. The vacuolating cytotoxin (*vacA*) is a well-established *H. pylori* virulence factor which has numerous impacts, including vacuolization of epithelial cells, the induction of apoptosis, increases in the permeability of epithelial monolayers, the formation of pores in cells and suppression of immune cell function [13].Gene encoding vacA (*vacA*) is available in all *H. pylori* strains and shows allelic diversity in three fundamental regions: the s(signal), the i(intermediate), and the m (middle) regions [13,14]. The s-region contains two main subtypes (s1 and s2). s1-region consist of three subtypes (s1a, s1b, and s1c), while m-region consist of two subtypes, m1 and m2 [15]. Various genotypic combination of *vacA* regions lead to different pathogenicity level as follows: s1a/m1 and s1b/m1 are considered the most virulent due to their production of high amount of toxin as compared to s1/m2 which produces moderate vacuolating toxins [16]. However, s2/m1 and s2/m2 are considered less toxic due to their inability to form vacuoles [9]. Previous Iraqi study estimated the relationship between specific *vacA* s and m

genotypes and the clinical outcome and it revealed that there was no association between *vacA* s and m genotypes types and clinical outcome [17]. Other Iraqi study revealed that no strong association was found between *vacA* alleles with the clinical outcome [18]. In Saudi Arabia, a study revealed that there was a significant correlation between the *vacA* s1/m2 genotype and gastritis cases and a significant correlation between *vacA* s1/m1 genotype and ulcer cases [19]. In Turkey, a study found that the *vacA* s1 allele was more frequently identified than the *vacA* s2 allele and there was significant association between *vacA* genotype and gastro-intestinal disease [20]. Iranian study did not find any relationship between *vacA* genotypes and clinical outcome among patients positive for *H. pylori* [21]. PCR technique performed on *H. pylori* DNA isolated from GTB sample and genotyping had been assessed previously.

Materials and Methods

Patients

In current cross section study which conducted from November 2015 to September 2016, GTB samples were obtained from 92 patients (50 male and 42 female) suffering from severe gastro-intestinal tract diseases (GIT), attended to under-went gastro-endoscopy and clinical examination at Gastrointestinal-tract and Liver Disease Hospital, Baghdad Medical City and Al-Imamein Medical City, Baghdad, Iraq. All patients were subject to the all tests included in the current study, but the results of only 69/92 patients were considered at the end of the current study, while 23 patients were excluded because their DNA samples had low concentration and quality.

From included patients,18/69 patients (17/18 patients with different gastro-pathologies not related to infection with *H. pylori* and 1/18 patient with intact mucosa according to histo-pathological examination), were considered to be as negative control for *H. pylori* after enrolled in identification methods of *H. pylori* that used in the current study (rapid urease test, histopathology examination and/or molecular identification of *ureA* and *vacA* genotyping) and they showed negative results.

Informed consent was obtained from each patient included in the study include name, age, gender, smoking and previous treatment were recorded. The endoscopic diagnosis was made by the consultant physician at the endoscopy department. Three GTB gastric tissues biopsies were obtained from the antral part of the stomach during gastrointestinal endoscopy. A rapid urease test was performed on one of the antral biopsies using HelicotecUT®Plus test Kit, the second biopsy was sent to histological lab and the third one was placed in 1 ml of normal saline and preserved at -20°C for molecular analysis.

Esophago-gastro-duodenal(OGD) finding were reported by specialist physicians. Gastric patients were grouped according to OGD finding as having chronic gastritis (CG), gastric ulcer (GU), duodenal ulcer (DU), gastric cancer (GC) and normal (intact mucosa). Histopathology examination results were taken from patient record. Patients with a history of gastric surgery, active gastrointestinal bleeding, whom had received antibiotics as proton pump inhibitors or bismuth compounds in the last four weeks were excluded. This study was approved by the ethical committee of the College of Medicine-Al-Nahrain University, Baghdad, Iraq.

Urease test

This test was done to detect the urease activity of *H. pylori* in gastric mucosal biopsies using HelicotecUT®Plus test Kit following manufacturer instructions.

Direct identification of H. pylori from GTB and detection of vacA genotypes using conventional PCR.

Genomic DNA was extracted from the GTB using WIZARD Genomic DNA Extraction Kit (Promega, USA) following manufacturer instructions. Concentration and purity of extracted DNA of each sample were measured using Nanodrop (*AcT* Gene NAS-99, USA). Extracted DNA with concentration (≥40 ng/µl) and purity between (1.8-2) were enrolled for farther estimations.

PCR was performed using specific primers for identification of species specific *ure*A in *H. pylori* [22,23] and to determine the frequency of *vacA* genotypes [24]. Target gene, amp icon size and primer sequences were shown in Table 1.

PCR master mix for each gene was prepared separately with total volume of 25 μl per reaction containing: 1X of PCR buffer, 200 μM of dNTPs, 20 *p*mol forward and reverse primers and 1.5 U of Taq DNA polymerase. Nuclease-free dd.H_2O was added until reach to the total volume of 23 μL. DNA (2 μL) was added to each reaction tube. No template control (NTC) tube was prepared for each master mix containing all PCR master mix components but instead of DNA, 2 μL of nuclease-free dd.H_2O was added. PCR reaction tubes were transferred to the thermal cycler (Eppendorf, Germany) and amplification program was performed as following: initial denaturation at 95°C for 3 min, then for 30 cycles, 95°C for 1 min, 45°C, 56°C or 57°C (depend on primer set) for 1 min and 72°C for 1 min. Final extension was done at 72°C for 5 min. PCR products were electrophoresed in 2% agarose gel.

Statistical analysis

Data were analyzed using SPSS version 22 and Microsoft Office Excel 2013. Categorical variables were presented as number and percentage while numeric variables were expressed as mean and standard deviation. Chi-square test was used to study association between any two Categorical variables. P-value was considered significant if equal or less than 0.05.

Table 1. *Primer sets used in this study.*

Target site	Primer and Sequences (5'-3')	Amplicon Size (bp)	Annealing temperature
UreA	HPU1-F GCCAATGGTAAATTAGTT HPU1-R CTCCTTAATTGTTTTTAC	411	45°C
*vacA*s1a	AA1-F GTCAGCATCACACCGCAAC AA1-R TGCTTGAATGCGCCAAAC	190	56°C
*vacA*s1b	SS3-F AGCGCCATACCGCAAGAG SS3-R CTGCTTGAATGCGCCAAAC	187	56°C
*vacA*s1c	S1C-F CTCGCTTTAGTGGGGCTA S1C-R CTGCTTGAATGCGCCAAAC	213	56°C
*vacA*s2	SS2-F GCTAACACGCCAAATGATCC SS2-RCTGCTTGAATGCGCCAAAC	199	56°C
vacA m1/m2	VAGFCAATCTGTCCAATCAAGCGAG VAG-R GCGTCTAAATAATTCCAAGG	570/645	57°C

Results

In the current cross-section study, 69 patients with symptoms of GIT diseases were enrolled. They were diagnosed as 33/69 (47.8%) patients with CG, 20/69 (28.9%) patients with DU, 14/69 (20.2%) patients with GU and 1/69 (1.4%) patients with GC, while 1/69 (1.4%) patient was diagnosed as intact mucosa. Among 69 gastric patients, 51/69 (73.9%) were positive for *H. pylori*. The remaining patients 18 (26.1%) were considered as negative control for *H. pylori*.

The mean age of 69 patients was 38.2 ± 17.09 years, ranging between 10-75 years. The mean age of patient's positive for *H. pylori* was 37.7 ± 15.6, ranging between 12-75 years. The mean age of patients considered as negative control for *H. pylori* was 39.72 ± 21.05. The age of majority of infected patients, 25/51 (49.01%) patients, was between (20-39) years followed by 13/51 (25.4%) patients between (40-59) years, 8 (15.6%) patients were at or older than 60 years and 5(0.9%) patients were less than 20. The number of male and female patient's positive for *H. pylori* were 28/51 (54.9%) and 23/51 (45.1%), respectively, with a ratio 1.21/1, (Figure 1). Three methods were used to identify *H. pylori* in GTB samples: RUT, histopathological examination and conventional PCR for the detection of *ure*A (Tables 2 and 3). Patients with two or more positive tests for *H. pylori* were considered positive. Using this assumption, 51/69 (73.9%) patients were identified as positive for *H. pylori* infection. Due to the high specificity and sensitivity of PCR technique, it was used as standard method to compare other tests used in current study (RUT and histopathology) in order to estimate specificity, sensitivity, positive predictive value and negative predictive value (Table 4).

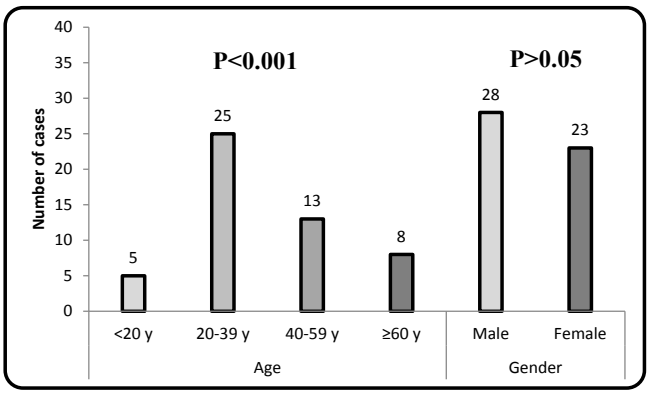

Figure 1. *Rate of infection according to age and gender.*

Table 2. *H. pylori identification methods.*

Identification test	No. (%)
Histology + RUT+PCR	15 (21.73)
RUT + PCR	39 (56.54)
Histology + PCR	15 (21.73)
Total	69 (100)

Table 3. *Rate of detection according to test used.*

Test	Patients No. (%)			Control No. (%)		
	Positive	Negative	Not identified	Positive	Negative	Not identified
Histopathology	26 (50.98)	0 (0.00)	25 (49.02)	0 (0.00)	4 (22.22)	14 (77.78)
Urease	39 (76.47)	0 (0.00)	12 (23.53)	0 (0.00)	14 (77.78)	4 (22.22)
PCR/ureA	51 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	18 (100.00)	0 (0.00)

All GTB samples (n=69) were investigated by PCR to detect the presence of *H. pylori* by using species-specific primer set for *ure*A, which revealed that 51/69 (73.91%) of samples were *H. pylori* positive (Figure 2 and Table 4).

In the current study, among 51 *H. pylori* positive GTBs, genotyping of s and m regions of *vacA* revealed that high frequency of *vacA* s1b as detected in 22/51 (43.14%) patients and s1a in 17/51 (33.3%) patients in compared to s2 which was detected in 12/51 (23.53%) patients, whereas s1c genotype was not detected (Figures 3 and 4). The *vacA* m2 was detected in 33/51 (64.71%) patients, in compared to *vacA* m1 which was detected in 15/51 (29.41%) patients (Figure 5). Only 3 GTBs were positive for vacA s but they were negative for *vac*A m, so they were excluded.

The distribution of various *vacA* allelic combinations in different disease groups shown in Table 5. The most frequent *vacA* allelic combination in patients with GU was s1b/m1 which detected in 4/14 (28.5%) patients followed by s1a/m1 which detected in 3/14 (21.4%) patients. Regarding to patients with DU, high frequency of s1a/m2 and s1b/m2 was detected in 5/20 (25%) patients and 4/20 (20%) patients, respectively. In patients with CG, high frequency genotype was s2/m2 which detected in 8/33 (24.24%) patients, while in GC patient, s1b/m1 genotype was detected. There was no statistically significant association between *vacA* genotypes and clinical outcome (P>0.05).

Discussion

Helicobacter pylori is one of the most prevalent bacteria known to infect human [25,26]. In the current study, 69 patients with symptoms of GIT diseases were included. The results of testing 53/69 (76.81%) GTBs using RUT revealed that 39/53 (73.58%) GTB were positive for *H. pylori*, while 14/53 (26.41%) GTB were negative. There is slightly difference between the result of the current study and previous Iraqi studies. One study found

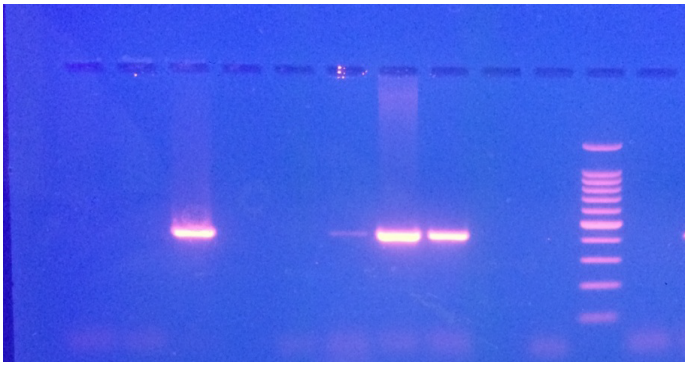

Figure 2. *Identification of H. pylori using species-specific primer for uerA. Lane (1,2,6): negative samples for uerA. Lane (3,7,8,): positive samples for uerA. Lane (5,10): NTC. Lane 11: DNA ladder (100 bp). Electrophoresis was done in 2% agarose gel at 5 V/cm for 60 minutes.*

Table 4. *Sensitivity of urease and histopathology.*

Value	Urease (%)	Histopathology (%)
Sensitivity	76.47	50.98
Specificity	100.00	100.00
PPV	100.00	100.00
NPV	40.00	41.86
Accuracy	82.61	63.77

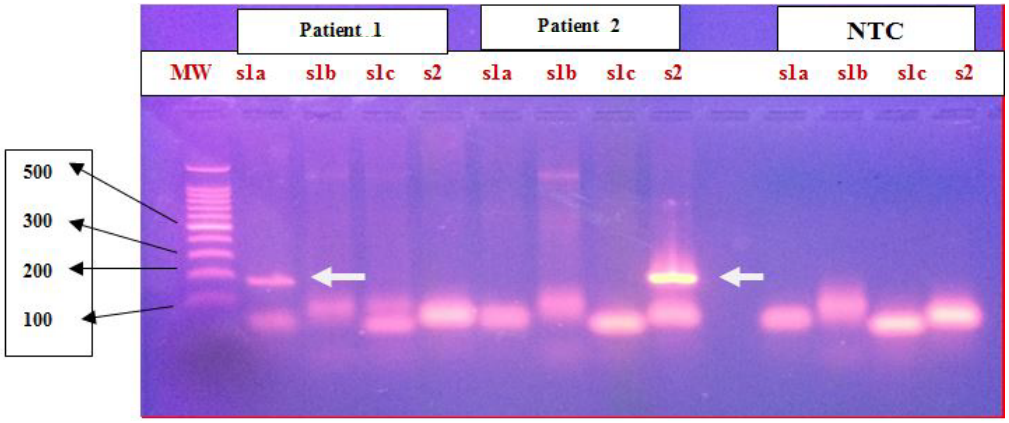

Figure 3. *PCR detection of vacA s genotypes. Lane (2,3,4,5): s1a, s1b, s1c, s2 respectively for patient no.1. Lane (6,7,8,9): s1a, s1b, s1c, s2 respectively, for patient no.2. Lane (11,12,13,14): NTC for s1a, s1b, s1c, s2, respectively. Lane 1: DNA ladder (100 bp). Electrophoresis was done in 2% agarose gel at 5 V/cm for 45 minutes.*

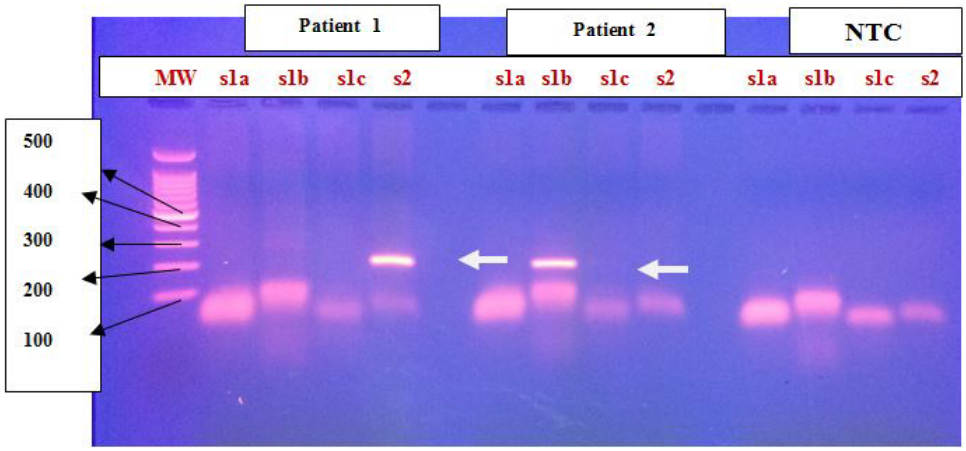

Figure 4. *PCR detection of vacA s genotypes. Lane (2,3,4,5): s1a, s1b, s1c, s2, respectively for patient no.1.Lane(7,8,9,10): s1a, s1b, s1c, s2, respectively for patient no.2. Lane (12,13,14,15): NTC for s1a, s1b, s1c, s2, respectively. Lane (1): DNA ladder (100 bp). Electrophoresis was done in 2% agarose gel at 5 V/cm for 45 minutes.*

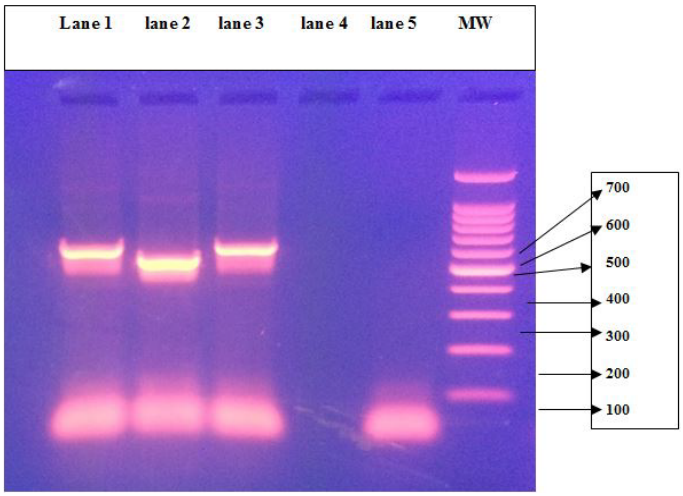

Figure 5. *PCR detection of vacA m (m1 570 bp/ m^2 645 bp) genotypes. Lane (1,3): vacA m^2 genotype. Lane (2): vacA m1 genotype. Lane (5): NTC. MW: DNA adder (100 bp). Electrophoresis was done in 2% agarose gel at 5 V/cm for 90 minutes.*

that 39/58 (67.2%) of gastritis patients were positive for *H. pylori* by using RUT [8]. In contract to Iraqi study which found that 102/210 (48.57%) GTBs were positive to *H. pylori* by using RUT [27]. Two Iranian studies recorded low percentage of positivity for *H. pylori* infection, 26/65 (40%) patients and 256/530 (48.3%) patients, respectively, using RUT [28]. But an Indian study revealed that 18/25 (81.8%) antral biopsy specimens collected from patients were positive for *H. pylori* using RUT [29].

The variation of *H. pylori* positivity by using RUT in different studies is due to the sensitivity of this test which depends on the type of urease media, concentration of urea, incubation

Table 5. *The distribution of various vacA allelic combinations in different disease groups.*

Description	CG	GU	DU	GC	total
ureA	23 (69.70)	9 (64.29)	18 (90.00)	1 (100.00)	51 (73.91)
S1a/m1	2 (6.06)	3 (21.43)	2 (10.00)	0 (0.00)	7 (10.14)
S1a/m2	4 (12.12)	1 (7.14)	5 (25.00)	0 (0.00)	10 (14.49)
S1b/m1	0 (0.00)	4 (28.57)	3 (15.00)	1 (100.00)	8 (11.59)
S1b/m2	8 (24.24)	1 (7.14)	4 (20.00)	0 (0.00)	13 (18.84)
S2/m1	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
S2/m2	8 (24.24)	0 (0.00)	2 (10.00)	0 (0.00)	10 (14.49)
Total	33 (100.00)	14 (100.00)	20 (100.00)	1 (100.00)	69 (100.00)

temperature, size of biopsy, buffering capacity, bacterial load and drug administration. Lack of attention to these factors could be lead to false results [30].

PCR has been used extensively for the identification of *H. pylori* from gastric biopsy specimens, saliva, feces and archival specimens [31]. PCR has also been found to be useful in detecting the organism when ordinary culture is difficult, as with testing environmental samples such as drinking water [32]. In the current study, a total of 69 GTB were tested by using PCR for identification of species-specific *ureA*. The results showed that 51/69 (73.9%) were positive for *H. pylori*. Also, species-specific *16SrRNA* was used for molecular identification of *H. pylori* but the results were not reliable so they were not presented. The frequency of identification of *H. pylori* defer from study to study depending on identification method used and the OGD of included patients. A study found that among peptic ulcer patients, 92/136 (67.6%) patients were positive for *H. pylori* infection using bacterial culture [33]. In other study, *H. pylori* was identified in 164/200 (82%) patients using histological examination [34]. Also, it was found that *H. pylori* was positive in 84/112 (75%) PUD patients using histological examination and stool antigen test [35]. The results of the current study agree with results of Turkish study which showed that 2353/3301 (71.3%) patients were *H. pylori* positive using histological examination [36]. Also, the results of the current study agree with the results of study conducted in China which found that 2310/3151 (73.3%) patients and 733/1022 (71.7%) patients were positive for *H. pylori* using serological testing and endoscopy, respective [37].

In the current study, high frequency of *H. pylori* may be attributed to many factors including inadequate living conditions, poor sanitation, hygiene and overcrowding and bacterial transmission via fecal-oral route. Several epidemiological studies have been reported the influence of particular virulence genes on clinical outcome of *H. pylori* infection in different geographic regions [18,38,39]. The current study was designed to characterize the more frequent *vac*A genotype in patients positive for *H. pylori* and possible association with OGD. The results of *vacA* genotyping of 51 GTB positive for *H. pylori* using conventional PCR showed higher percentage of m2 genotype which found in 33/51 (64.71%) patients, than m1 genotype which found in 15/51 (29.41%) patients. The more frequent *vacA* s genotype was *vacA* s1 with its both subtypes s1b and s1a which found in 22/51 (43.14%) patients and 17/51 (33.33%) patients, respectively, while *vacA* s2 genotype found in 12/51 (23.53%) patients. Also, it was found a high frequency of *vacA* s1 genotype as compared to *vacA* s2, and the high frequency of *vacA* m2 as compared to *vacA* m1 genotype. That agree with the results of previous Iraqi study which found that *vacA* allelic types distributed among *H. pylori* as 28/30 (93.3%) isolates were s1 and 15/30 (23.3%) isolates were m1 [39]. Other Iraqi study found among 63/114 (55.3%) patients positive for *H. pylori*, the percentages of s1, s2, m1 and m2 were 57%, 43%, 46% and 54%, respectively [18]. The percentages of allelic genotypes that found in the current study agree with the results of Iranian study, which revealed that *vacA* s1 versus *vacA* s2 was (76.9 vs. 23.1%), and *vacA* m1 versus *vacA* m2 was (32.5 vs. 66.5%), among 207 samples from *H. pylori* positive infected dyspeptic patients [40].

The similarity of the genotypes identified in the three neighboring countries Iraq, Iran and Turkey could be explained by a primary geographic influence important in the adaptation of the organism to the environment and climatic conditions, despite the obvious host differences in life style in the neighboring countries. The close resemblance of strains in neighboring countries was also reported in Bangladesh and India, which was quite likely considering the close proximity of the two countries, the similar physiological environments, and life styles of the host [41,42].

In the current study, the identification of high frequency of *vacA* s1b is agree with that reports from Portugal, Central and South America and Latin America [43,44]. Regarding *vacA* s1c, it was not determined in current study and that agree with Turkish studies [20,38]. In contrast with studies from East Asia and Japan, they showed that the s1c was the more predominant *vacA* genotype [43,45,46].

In the current study, the most predominant *vacA* genotypes combination was s1b/m2 which found in 13/69 (18.84%) patients followed by 10/69 (14.49%) patients for each of s1a/m2 and s2/m2, 8/69 (11.59%) patients for s1b/m1 and 7/69 (10.14%) patients for s1a/m1. That agree with the findings of the studies conducted in Saudi Arabia [19], Turkey [47] and Iran [21,48], where the s1/m2 genotype combinations was the most common. In contrast to the study done in Thailan which reported that the most predominant *vacA* was s1/m1 genotype [49]. The differences in percentages due to the variations in the number of patients included in each study.

In the current study, the examination of the association between *vacA* s (s1a, s1b, s1c, s2) and m (m1, m2) combination and clinical outcome revealed that there was no statistically significant association. That agree with results of previous Iraqi studies which revealed that there was no association between *vacA* s and m genotypes and clinical outcome [17,18]. Also, studies from Jordan, Kuwait and Iran found no association between *vacA* s and m genotypes and clinical outcome [50,51].

Because of the low sample power, it was unclear whether the connection between virulence factors and disease development has an association.

Conclusions

In conclusion, there is no statically significant relationship between the genotype of *H. pylori* and the development of clinical outcomes in infected patient.

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