

Prevalence of *vacA*, *cagA*, and *iceA* Virulence Factors of *Helicobacter Pylori* Isolated from Gastro-duodenal Patients

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Abstract

Back ground: *Helicobacter pylori* are bacteria colonize in the human epithelial cells of the gastrointestinal tract. Its infection causes different diseases, including chronic gastritis, peptic ulcers, gastric lymphoma and adenocarcinoma. *H. pylori* have many virulence factors attributing in one or more biological functions.

Objective: Detecting the prevalence of virulence factor genes *vacA*, *cagA*, *iceA* among strain of *H. pylori* using molecular technique (PCR).

Materials and methods: Sixty patients (27 male and 33 female), aged 18 and above included in the present study who showed signs and symptoms of *H. pylori*, and undergo endoscopy between period of November 2019 and February 2020. RUT and PCR test done to detect the presence of *H. pylori* infection, also PCR used to detect the three virulence factors.

Results: Result showed that 44 patients, 21 (47.7%) male and 23 (52.3%) female were detected as positive *H. pylori* infections, among them 13 (29.5%) above 50 years, and 31 (70.4%) were below 50 years. While prevalence of the virulence factors *vacA*, *cagA*, and *iceA* were (100%), (84.1%), and (34.1%) respectively.

Conclusion: It can be concluded that the frequency and prevalence of these genes are differed and showed significant differences among them. Also, PCR test is sensitive and accurate for detection of *H. pylori* virulence genes.

Keywords: *Helicobacter pylori*, Virulence Factors, CagA, VacA, IceA, PCR.

Introduction

H. pylori is a usually spiral-shaped, gram-negative bacterium, its length is 2 to 4 μm and 0.5 to 1 μm in width with lophotrichous flagella (1,2). *H. pylori*'s prevalence shows large geographical variations worldwide (3,4). *H. pylori* infection causes peptic ulcer, chronic gastritis, mucosa-associated lymphoid tissue lymphoma, and gastric carcinoma (5,6). *H. pylori* have different virulence factors that perform different biological functions, some virulence factors have subtypes also (7,8). The virulence factors of *H. pylori* can be categorized to three major pathogenic processes, including colonization, immune escape, and disease induction (5,9).

Vacuolating cytotoxin a (*VacA*) is a cytotoxin that secreted from *H. Pylori* as a large polypeptide, and before delivering to the host cells as an active form it must be modified by trimming its both ends to be able to exerts its activity (10,11). It performs biological functions like: phagocytosis suppression, inducing tolerogenic dendritic cell, and blocking of effector T-cell response. In addition, *vacA* has an important role for colonization of *H. pylori* in vivo. (5,9). All *H. pylori* strains have the gene that encode *vacA*, although the cytotoxic activity of the toxin varies among strains by displaying allelic diversity in three main regions of the gene which are the s (signal), the i (intermediate), and the m (middle) regions (12).

Cytotoxin-associated gene a (*CagA*) is one of the most important virulence factors of the *H. pylori* that have many biological functions including: phagocytosis suppression, decreasing antimicrobial peptide, inducing tolerogenic dendritic cell, and blocking of effector T-cell response (9). Beside those functions, *cagA* also related to other diseases and consequence, it could have a relation even with infertility in men (13). Not all strain of *H. pylori* have *CagA* virulence factor, strains can be divided into two types, those who have this virulence factor considered as *CagA* positive, while those who lack it are *CagA* negative strains. The

geographical distribution differs for these strains, in Western countries, approximately 60% of *H. pylori* strains carry CagA, while in East Asian, almost all of the isolates are CagA positive (14, 15).

Induced by Contact with Epithelium (*iceA*) gene was identified when investigating genes “induced by contact of *H. pylori* with epithelium” in 1998 (9). There is an association between the presence of *iceA* and peptic ulcer disease (PUD), also, *iceA* is related with other clinical outcomes in the host, like acute antral inflammation and enhancing the expression of mucosal interleukin (IL-8) (8, 16).

Different diagnostic methods used to detect *H. pylori* infection. Among them, PCR test show a high accuracy in detecting *H. pylori* and genes of virulence factors. PCR has superiority on other diagnostic methods as it is simple, rapid and it is very accurate in detecting different genes and their mutations (5, 17). In Iraq there is little attention about the genotyping of *H. pylori* isolate therefore this study aimed to detect *h. pylori* using PCR test, and to identify the prevalence of *H. pylori vacA*, *cagA*, and *iceA* virulence factors among gastrointestinal patients from Erbil city.

Materials and Methods

Sample collection

The present study included 60 samples that collected at Rizgary Teaching Hospital defectively at Hawler Gastroenterology and Hepatology Center. Samples collected from period of November 2019 and February 2020. Patients included 27 male, 33 female aged 18 and above. All samples collected from patients who submitted to endoscopy procedure. Patients followed instruction before taking biopsy samples like elimination of food and drug taking a day before the procedure. Two samples of 0.5 cm of the biopsy collected in two different tubes, The first tube used for RUT, while the second tube that contain Dulbecco's phosphate-buffered saline (DPBS) used for PCR test.

DNA extraction

Genomic DNA extracted directly from biopsy samples using the Presto™ Mini gDNA Bacteria Kit following the manufacturer's instructions; 100 µL elution buffer was used for extracting. Extracted genomics was stored at -20 °C before running PCR (18).

Estimation of Extracted DNA

Before PCR run, agarose gel electrophoresis used to estimate the extracted genomic matter of *H. pylori*. In which 1% agarose gel used and run on 85V for 45min.

Polymerase chain reaction (PCR)

Primers

In the present study five sets of primers were used. The first primer 16s rRNA used to detect the genome of *H. pylori*. The other four primers used to detect genes of virulence factors (*vagA*, *cagA*, and *iceA*). All primers sequences and the size of product are listed in Table (1):

Table (1): the six primers used in present study

Primers	Sequence of the primers (5' to 3')	Size of the product by base pairs
<i>CagA</i>	Forward: TGCTAAATTAGACAATTGAGCGA	290 bp.
	Reverse: AATAATCAACAAACATCACGCCT	
<i>VacA</i>	Forward: GCGGTATCAATCTGTCCAATCA	68 bp.
	Reverse: TGATATTCCCGTTAGATTTTCCA	
<i>iceA</i>	Forward: GACAAGCGGTTGGAGTTTGC	851 bp.
	Reverse: GAGATCATGGCCTACAACCG	
<i>16s rRNA</i>	Forward: ACTCCCTACGGGGAAAGAT	141 bp.
	Reverse: GGACCGTGTCTCAGTTCCAG	

PCR run and condition

Selected genes were amplified using DNA amplification with some optimization, cycling was carried out (Gradient thermal cycler Alpha Cycler PCRmax series). The primers used for PCR were specific for the detection of *H. pylori* DNA and the four virulence factors (see Table1). Mixture total of 25 μ l used for each primer in 0.5 ml microcentrifuge tube containing: 12.5 μ l master mix, 2.5 μ l forward primer, 2.5 μ l reverse primer, 3 μ l of the extracted genome, the volume completed to 25 μ l by adding 4.5 μ l of free nuclease water (19).

PCR condition and cycling were same for amplification of all the primers, in which started with initial denaturation 95°C for 5 minute followed by 45 cycles of denaturation 95°C for 40 second, annealing 59°C for 45 second, extension 72°C for 40 second, and followed by final extension 72°C for 5 minute.

Gel electrophoresis

After running the PCR for each sample using each primer separately, gel electrophoresis done for each sample to observe the results of the amplification, put in the tank and filled with TAE buffer, then add 6 μ l of the PCR product to each well (note: no need to add loading dye because the master mix already contain it in their component). After loading the samples, the tank cover placed and the electric pole wires (+ and – poles) of the electrophoresis gel apparatus connected to its specific places. Power supply of 45 volts was applied for 10 minutes until the DNA left the wells and moved toward the positive electrical side, then the voltage increased to 75 v/45min. Results of amplified DNA bands were visualized by UV-light illuminator at (240,366nm) wave length. Pictures of the gel documented using 16MPX camera photography.

Statistical analysis of the results

Chi-square and Fisher's exact test used to analyze the results of the present study with ($P < 0.05$) for significant differences.

Results

Gastric biopsies of sixty patients were analyzed, in which 44 samples showed positive results for *h. pylori* infection, 21 (47.7%) male and 23 (52.3%) female. Participant ages varied from 18 to 71 years, in which 13 of them were 50 years or older. Results of all participants regarding to gender and age are shown in Table (2).

Table (2): The age and gender of the 44 positive *H. pylori* patients approved by PCR test in the present study

Variable	Number (n.)	Percentage %
Gender (n = 44)		
Male	21	47.7
female	23	52.3
Age (n = 44)		
Above 50 years	13	29.5
Below 50 years	31	70.5

Regarding results of PCR test using 16srRNA for detecting the presence of *h. pylori* genome among 60 biopsy samples showed 44 (73.3%) as positive, and 16 (26.7%) as negative, as shown in Figure (1).



Figure (1): Agarose gel electrophoresis analysis 1% (stained with safe stain) of PCR products presenting partial specific primer (16srRNA) that showed the expected size 141 bp. Lane L: is 100 bp DNA Marker; Lane 2 is (-ve) negative control, Lanes 3-16: samples amplified from *h. pylori* genome run on 75V for 45min. samples number 1 to10, 14, and 15 were positive for *H.pylori* infection, while samples number 12, 13 and 16 were negative.

Results of PCR test using specific primer to detect *vacA* gene among 44 positive *h. pylori*, showed 44 (100%) positive, as shown in Figure (2). PCR test for *cagA* gene showed 37 (84.1%) positive results, as shown in Figure (3). And finally, PCR test for *iceA* gene showed 15 (34.1%) positive results, as shown in Figure (4).

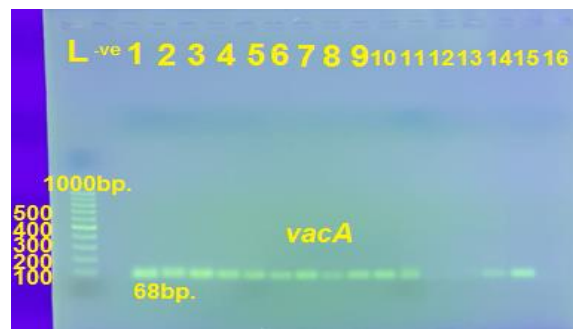


Figure (2): Agarose gel electrophoresis analysis 1% (stained with safe stain) of PCR products using specific primer (*vacA*) that showed the expected size 68 bp. Lane L: is 100 bp DNA Marker; Lane 2 is (-ve) negative control, Lanes 3-16: samples amplified from *H. pylori* genome run on 75V for 45min. samples number 1 to10, 14, and 15 were positive for *vacA* gene, while samples number 12, 13 and 16 were negative.

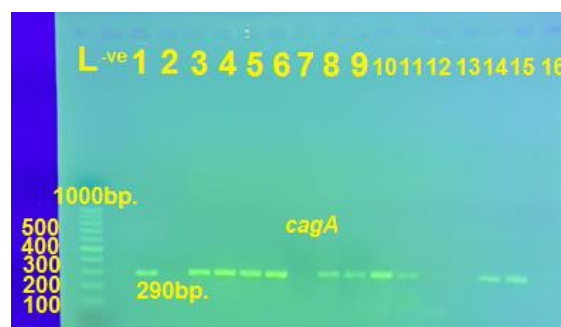


Figure (3): Agarose gel electrophoresis analysis 1% (stained with safe stain) of PCR products using specific primer (*cagA*) that showed the expected size 290 bp. Lane L: is 100 bp DNA Marker; Lane 2 is (-ve) negative control, Lanes 3-16: samples amplified from *H. pylori* genome run on 75V for 45min. samples number 1, 3, 4, 5, 6, 8, 9, 10, 11, 14 and 15 were positive for *cagA* gene, while samples number 2, 7, 12, 13 and 16 were negative.



Figure (4): Agarose gel electrophoresis analysis 1% (stained with safe stain) of PCR products using specific primer (*iceA*) that showed the expected size 851 bp. Lane L: is 100 bp DNA Marker; Lane 2 is (-ve) negative control, Lanes 3-16: samples amplified from *H. pylori* genome run on 75V for 45min. samples number 4, 6, 8, 10 and 14 were positive for *iceA* gene, while samples number 1, to 3, 5, 7, 9, 11, to 13, 15 and 16 were negative.

The overall result of PCR test for detecting genes of virulence factors *vacA*, *cagA*, *iceA* among 44 positive cases approved by PCR were 44 (100%), 37 (84.1%), and 15 (34.1%) respectively as shown in Figure (5).

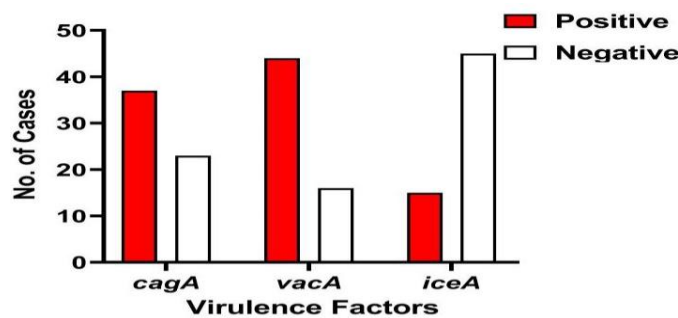


Figure (5): number of *vacA*, *cagA*, *iceA* genes out of 44 positive PCR cases

Discussion

The present study included 60 patients in which 44 samples showed positive results for *H. pylori* infection. 21 (47.7%) of them were males and 23 (52.3%) females. Result of statistical analysis showed non-significant differences with ($P < 0.56$) regarding gender of the patients.

Regarding *vacA* gene, among 44 positive *h. pylori* patients, all patients (100%) showed positive results for this virulence factor gene. Detecting this gene in all *H. pylori* strains is normal as all *H. pylori* strains have the gene encode *vacA* (12). According to a recent study carried out in 2019 in Saudi Arabia, that included samples of gastric biopsies, detected *vacA* in all included samples with (100%) frequency, this approves and agree with present study (20). Also, another research carried by Flores- Treviño and his colleagues detected *vacA* gene in all samples that were 16srRNA positives for *H. pylori*, which 100% agree with current study (21). Other researches detected *vacA* in lower frequencies, for example, Idowu *et al.* detected *vacA* with 90.6% (22), the explanation of such differences belong to investigating subtypes of *vacA*, unlike present study which used a primer for *vacA* gene in general.

The present study detected *CagA* gene in 37 (84.1%) out of 44 positive PCR cases. According to a research carried out in 2017, detected *cagA* in 77% of the samples which somehow agree whit the present study (23). In an approach to detect prevalence of *cagA* in Kurdistan region, (24) and his colleagues in 2013 detected *cagA* with (72%) frequency. Another research carried by (25) detected *cagA* with (72%) frequency, having such differences in frequency of *cagA* will be clear considering geographical distribution of these researches which has an impact on the types of *H. pylori* strains (26).

The last virulence factor gene *IceA*, detected in 15 (34.1%) among 44 positive cases. Results of (20) for detection of *iceA* gene with approximately same frequencies agree with results of present study.

Another research carried out in Egypt, detected *iceA* with (38.8%) frequency that agree with the present study (27). Detecting *iceA* gene in lower frequencies than *vacA* and *cagA* may be due to the source of clinical isolates, as *vacA* and *cagA* are more contributed to gastric disease than *iceA*, also *iceA*, is found to be independent of *cagA* and *vacA* (6).

Finally, the studied genes of virulence factors in the present study showed agreement and differences with other studies. Several factors play role in differences like; geographical distribution, sample size, time of sampling and source of the clinical samples. Generally, genotyping of *H. pylori* strains obtained from multiple human populations around the world has demonstrated that the genetic diversity of this bacterium reflects human migrations and subsequent geographic and ethnic separation between human groups (28).

Conclusion

It can be concluded that genes of virulent factors showed variability and different frequencies among *H. pylori*. Some virulence factors present in all strain of the bacterium while other not. The frequency of the virulence factors depend on its biological functions. Geographical distribution also plays an important role in the distribution of those virulence factors.

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