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Molecular detection of some virulence gene of Helicobacter pylori isolated from patients with gastroenteritis in Al-Diwaniyah Governorate/Iraq

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***Abstract*---**Infection with *Helicobacter pylori* is one of the most common chronic bacterial infections worldwide. So, This study aimed to detect *Helicobacter pylori* in patients with gastroenteritis symptoms by molecular method Polymeraee chain reaction (PCR) . This study was conducted in AL- Diwaniyah province/Iraq and included 40 patients with age ranged from 11 to 56 years and they were males and females. The Physician diagnosed patients with stomach and intestinal infections. Samples were collected from the Consultant Internal Medicine and the Division of Gastroenterology at Al-Diwaniyah Teaching Hospital during the period from October 2021 to March 2022 . Tissue biopsy samples were collected from patients suffering from gastroenteritis in the Gastrointestinal Division. Three tissue biopsies were removed from the gastric antrum of each patient using forceps for endoscopy. The total number of samples was 20. Also, 20 Stool samples were collected from patients and then placed in a sterile container. *Helicobacter pylori* were diagnosed according to the diagnostic gene 16SrRNA in 12 samples with a percentage of 30% .The diagnosis of these positive samples was confirmed by a number of virulence factors: cytotoxic associated gene A protein (*cag A)*, Vacuolating Cytotoxin gene Type A (*vacA S1/S2)* , Induced by Contact with Epithelium Gene A (*IceA1)* and *Urease A*, with percentages of (100%, 100%, 58.33%, 100%, respectively) . In conclusion, the results detect the effectiveness of PCR in diagnosis of *Helicobacter pylori* based on housekeeping gene and virulence genes.

***Keywords*---**gastroenteritis, Helicobcter pylori, molecular detection, virulence factors**.**

Introduction

Helicobacter pylori(*H.pylori*) ,Foremerly known as *Campylobacter pylori*(Waite *et al.*, 2017), is a gram-negative bacterium that infects large numbers of people in the world, where It infects the epithelium of the stomach of humans. The infection rate ranges between 30% and 50% in developed countries and reaches 80% in developing countries, especially in Asia (Eusebi *et al.*, 2014; Mentis *et al.*, 2015) . It is a microaerophilic bacterium and has a curved or S-shaped, 2 to 4 μm in diameter and 0.5 to 1 μm in width, a flagellated, and none spore forming bacterium (Momtaz *et al.*, 2014; Mousavi *et al.*, 2014; Ranjbar *et al.*, 2018) . There is gradual damage to the gastric mucosa which can be caused by *Helicobacter pylori* infection, and there are many diseases that are associated with this bacteria, such as peptic ulcers, gastritis, stomach cancer (Yamaoka & Graham, 2014) . Serious diseases such as stomach cancer generally occur after decades of chronic infection with bacteria. Despite the harsh conditions in the stomach environment of low pH and variable conditions, *Helicobacter pylori* evolved to colonize the stomach continuously, indicating that it has a strong ability to adapt (De la Cruz *et al.*, 2017) . There are many virulence factors in these bacteria, such as the cytotoxin A-related gene (*CagA*), the vacuum-forming cytotoxin (*Vac A*), and they also produce the enzyme urease that breaks down urea in the medium to ammonia, which has an antacid effect on the stomach lining (Bakir, 2018) . Depending on the presence or absence of a *cag* pathogenicity island (cagPAI), *H. pylori* can be distinguished into cagA+ and cagA-strains that encode a type IV secretion system and in which the effector is *CagA* (Hashi *et al.*, 2018) . The *cagA* gene is often used as a measure of *Helicobacter pylori* virulence activity due to its role in increasing IL8 production and activation of nuclear factor-kB, as it is said to be responsible for mucositis and cancer development (Abu-Taleb *et al.*, 2018) .*VacA* is present in all strains of *Helicobacter pylori*. It is a pore-forming, high-molecular-weight protein unlike any known bacterial or eukaryotic protein. It persists in the stomach through suppression of macrophages and T cells, formation of vacuum-like membrane vesicles in the cytoplasm of gastric cells, and induction of apoptosis (Ali *et al.*, 2015; Weng *et al.*, 2019) . The *iceA* gene was discovered in *H. pylori* isolates from individuals with PUD and gastritis. *IceA* has at least two alleles, *iceA1* and *iceA2* (Subsomwong *et al.*, 2017) . When *H. pylori* came into contact with human epithelial cells, the expression of *iceA1* increased. The *iceA1* genotype was linked to increased mucosal IL-8 expression and acute antral inflammation. Furthermore, adhesion to gastric epithelial cells in vitro promotes *iceA1* transcription(Šterbenc *et al.*, 2019) .There are a number of methods for diagnosing bacterial infection, and they are classified as invasive or non-invasive. Preference is given to non-invasive diagnostic methods. Invasive methods require upper endoscopy and gastric biopsy analysis. If the patient requires upper endoscopy, it can be diagnosed by histological analysis, rapid urease test, molecular methods, or culture to diagnose *H. pylori* infection (Ricci *et al.*, 2007; Pichon *et al.*, 2020) . Molecular diagnostic methods using conventional polymerase chain reaction (PCR) or real-time polymerase chain reaction (RT-PCR) , rely on DNA amplification through stool, saliva ,and stomach biopsy that can detect the genetic material (DNA) of bacteria. Depending on the applied substance, polymerase chain reaction (PCR) can be considered an invasive or non-invasive method for the detection of *Helicobacter pylori* . It shows sensitivity of up to 95% and specificity of 95% (Szymczak *et al.*, 2020) . PCR allows detection

of bacterial virulence factors such as *CagA* and *VacA*, and specific mutations that lead to antibiotic resistance. *Helicobacter pylori* has a large number of housekeeping genes that might be used for bacterial genetic diagnosis. The most frequent housekeeping genetic marker is the 16SrRNA gene, and amplification of the 16SrRNA gene has been proven to be beneficial for bacterial identification and phylogeny (Hackett & Preston, 2021) . So, The aim of this work is to detect some virulence genes of *Helicobacter pylori* in patients with Gastroenteritis symptoms using the molecular method (PCR).

Materials and Methods

Patients: This study included 40 patients from both sexes in Al-Diwaniyah province / Iraq. The age of patients were ranged from 11 and 56 years . Samples were collected from October 2021 to March 2022. The physician diagnosed patients with stomach and intestinal infections.

Samples collection :Samples were collected from the Consultant Internal Medicine and the Division of Gastroenterology at Al-Diwaniyah Teaching Hospital from both sexes. Tissue biopsy samples were collected from patients suffering from gastroenteritis in the Gastrointestinal Division. Three tissue biopsies were removed from the gastric antrum of each patient using forceps for endoscopy. Xylocaine for local anesthesia and midozolem was used. The total number of samples was 20 biopsies .Also, 20 Stool samples were collected from patients and then placed in a sterile container. Biopsy samples were kept in normal saline, and stool samples were kept in phosphate-buffered saline. Then all samples were kept by deep freezing (-70°C) in the hospital until examination by PCR .

Molecular detection of *Helicobacter pylori* by 16SrRNA gene

DNA extraction

DNA extracted from Biopsy tissue and stool samples by using G-spin™Total DNAExtraction Kit(iNtRON/Korea) and Presto™ Stool DNA Extraction Kit(Geneaid/USA) respectively. The extraction was done according to the protocol of the manufacturer's instructions . Quality and quantity of DNA was measured by a NanoDrop(Thermo / USA) .

Amplification of 16SrRNA gene and virulence genes by PCR

PCR master mix reactions were prepared for all genes using the GoTaq® Green PCR master mix kit(Promega/USA), and this master mix was done according to the company's instructions. The total volume of the reaction was 25 µL that includes 5µL DNA template (5-50 ng) ,2µL Forward primer (10pmol), 2µL Reveres primer (10 pmol) ,12.5µL of GoTaq® Green PCR master and 3.5µL of PCR water . The primer was designed based on references (Table 1) and NCBI-Genbank . PCR thermocycler conditions protocol for each gene was calculated by using Optimase Protocol Writer™ online application and done by using Touchdown protocol for 16SrRNA gene and some of virulence genes; *VacAs1/s2* , *IceA1* and *Urease A* (Table 2) and conventional PCR thermocycler for *cagA* gene(Table 3) .

Table (1) PCR Primers used in this study

Target gene	Primer pair (5′-3′)	Product Size(bp)	References
16SrRNA	F:CTGGAGAGACTAAGCCCTCC R:AGGATCAAGGTTTAAGGATT	446	(Singh *et al.*, 2008)
CagA	F: CAAGCCCTAGCCGAACTCAA R: GCTTCTGACACTGCCTGACT	599	NCBI-Genbank
VacAs1/s 2	F:ATGGAAATACAACAAACACAC R: CTGCTTGAATGCGCCAAAC	259/286	(Idowu *et al.*, 2019)
Urease A HPUgene	F: GCCAATGGTAAATTAGTT R: CTCCTTAATTGTTTTTAC	411	(Ashton-Key *et al.*, 1996)
IceA1	F:CGTTGGGTAAGCGTTACAGAATTT R:TCATTGTATATCCTATCATTACAAG	558	(Essawi *et al.*, 2013a)

Table (2)Touchdown protocol of 16SrRNA gene

Touch Down PCR step	Temp.	Time	repeat
Initial Denaturation	95°C	2min.	1
Denaturation	95 °C	30sec.	14
Annealing	57.1 °C decrease 0.5 per cycle	30sec	
Extension	72 °C	50sec	
Denaturation	95 °C	30sec.	19
Annealing	50.1 °C	30sec	
Extension	72 °C	50sec	
Final extension	72 °C	5min.	1
Hold	4 °C	Forever	-

Touchdown protocols of other genes was the same of 16SrRNA gene with some changes in some steps, which are for *VaAcs1/s2* (Annealing step : 58.3 °C, 51.3 °C), *IceA1*(Annealing step: 60.5 °C, 53.5 °C), *ureaseA* (Annealing step; 50.0 °C, 43.0 °C).

Table (3) PCR Thermocycler conditions of *cagA* gene

PCR step	Temp.	Time	Repeat
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	30sec.	35 cycle
Annealing	58°C	30sec	
Extension	72°C	1 min	
Final extension	72°C	5min	1
Hold	4°C	Forever	-

PCR product analysis

The Agarose gel electrophoresis was used to analyze the products of PCR.Where ,1.5 % agarose gel including 0.5µg/ml Ethedium bromide was used and DNA

bands that were separated via electrophoresis and visualized by U.V Transilluminator (Wisd/Korea).

Results

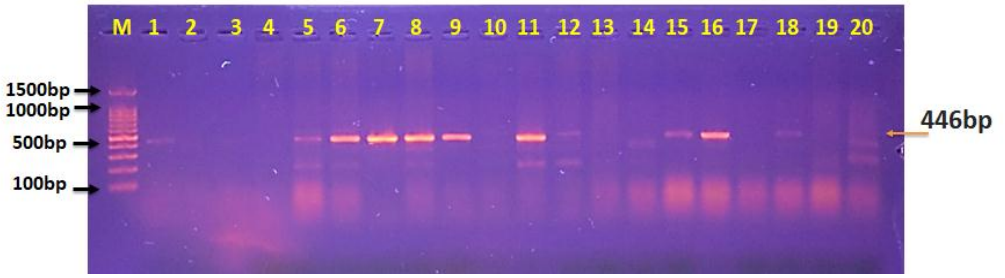
Identification of *Helicobacter pylori* by biochemical and molecular assays

The traditional biochemical tests such as urease were used to identify *Helicobacter pylori* (MacFaddin, 2000) , all the 20 biopsies samples were diagnosed as *Helicobacter pylori* . While the *H. pylori* antigen test kit (high top / China) used for stool samples indicates that all 20 samples showed positive results. Polymerase chain reaction (PCR) technique was used to diagnosis bacteria after extracting DNA from biopsy and stool samples and DNA amplification, it was diagnosed by the 16SrRNA gene, where 12 out of 40 samples were diagnosed as *H.pylori* with a rate (30%).

Detection of virulence factors genes : The 12 positive isolates of *H.pylori* diagnosed by 16SrRNA were used to detect some virulence factors genes that appeared with different ratios as mentioned in table(4) and figure(1) to figure (5).

Table (4) The rate (%) of virulence factors genes in *H.pylori* isolates

Genes	Positive samples	%
CagA	12	100
VacAS1/S2	12	100
UreaseA	12	100
IceA1	7	58.33



Figure(1): Image of agarose gel electrophoresis showing the product of PCR of 16SrRNA gene of *Helicobacter pylori* with product size 446bp .Where Marker ladder(100-1500bp), lane (1-20): Isolates numbers.

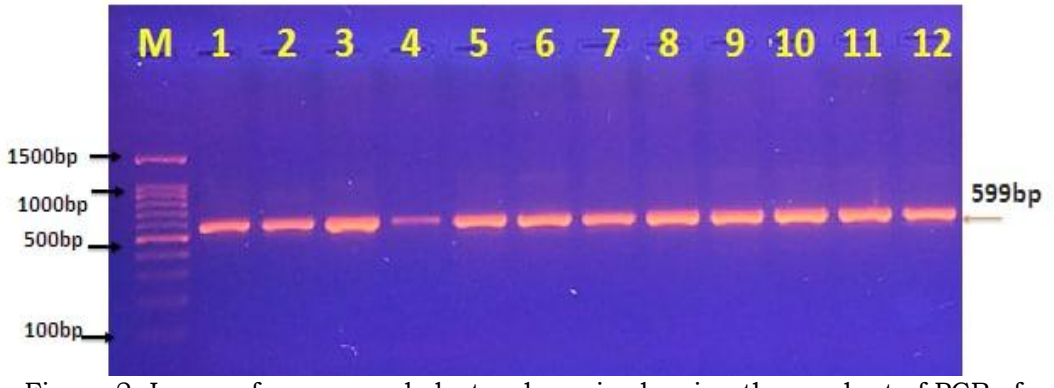


Figure 2: Image of agarose gel electrophoresis showing the product of PCR of virulence factor gene *cagA* of *H. pylori* with product size 599 bp .Where Marker ladder (100-1500bp), lane (1-12): Isolates numbers

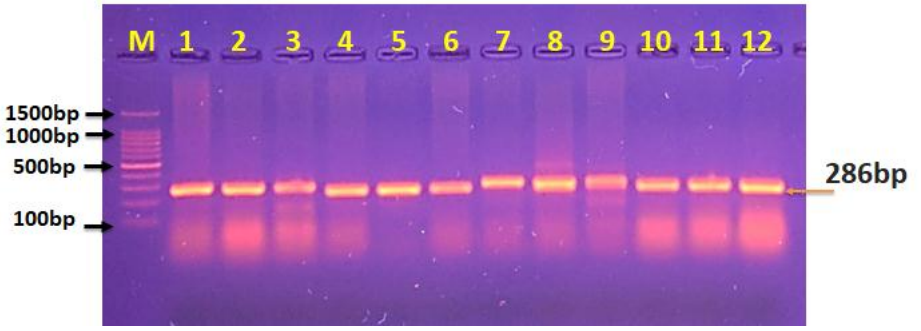


Figure 3: Image of agarose gel electrophoresis showing the product of PCR of virulence factor gene *vacAs1/s2* of *H. pylori* with product size 286 bp .Where Marker ladder (100-1500bp) , lane (1-12): Isolates numbers

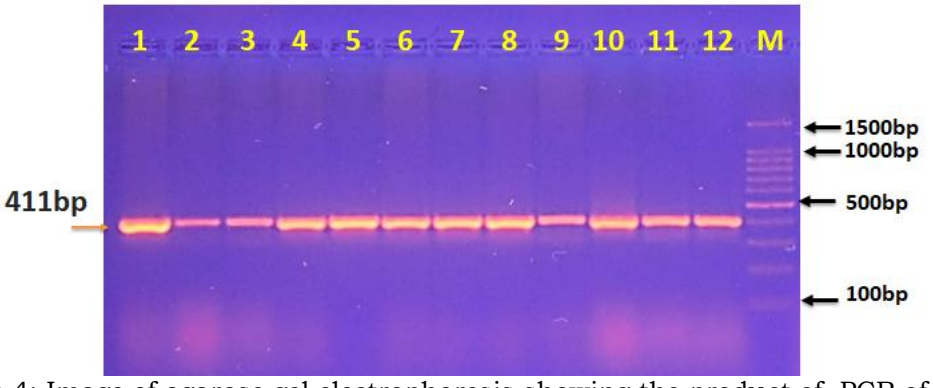


Figure 4: Image of agarose gel electrophoresis showing the product of PCR of virulence factor gene *Urease A* of *H. pylori* with product size 411bp .Where Marker ladder (100-1500bp), lane (1-12): Isolates numbers

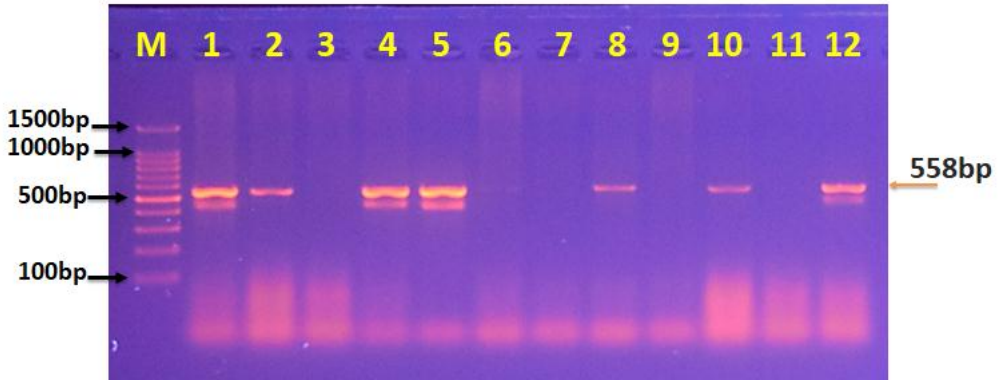


Figure 5: Image of agarose gel electrophoresis showing the product of PCR of virulence factor gene *IceA1* of *H. pylori* with product size 558 bp .Where Marker ladder (100-1500bp), lane (1-12): Isolates numbers.

Discussion

Our study included the diagnosis of *H. pylori* bacteria in patients who suffer from gastroenteritis, where tissue biopsy samples were taken from them .*H. pylori* has a large number of housekeeping genes that might be used for bacterial diagnosis. The most frequent housekeeping genetic marker is the 16SrRNA gene, and amplification of the 16SrRNA gene has been proven to be beneficial for bacterial identification and phylogeny (Hackett & Preston, 2021) . The diagnosis was made by traditional biochemical test including a urease test for biopsies samples and then confirmed by the diagnostic gene 16SrRNA, after which positive samples were screened to detect some virulence factors genes; *cagA*, *I ceA1*, *ureaseA*, and *vacA s1/s2* . The results of this study showed the diagnosis of *H. pylori* in 12 samples, where 30% gave a positive result, while it was diagnosed in another study by 44% of the 100 samples included in a study conducted by (Essawi *et al.*, 2013) . In another study by (El-Shenawy *et al.*, 2017), bacteria were diagnosed in 60 samples, and its rate was 53.1%. Researchers in a study showed that the results of diagnosing bacteria by this technique were only 48.9 percent, a higher percentage than what was diagnosed in our study (Idowu *et al.*, 2019) . A study was conducted in South Africa by a number of researchers (Tanih *et al.*, 2010) . Positive samples were diagnosed based on the VacA s1/s2 gene from the patients included in the study, and the percentage of positive samples was 16%. These results differed from ours in that they were 100% diagnosed . In our study, *cagA* was diagnosed in 12 positive samples with a percentage of 100%, while in other study by(Khiddi *et al.*, 2020) , it was diagnosed at 30.26 percent . *CagA* was diagnosed by a number of researchers at a rate of 26.6 % where they differed with our study, the virulence factor was diagnosed by 100% . The researchers also diagnosed the virulence factor *iceA1* at 38.3 percent, while it was diagnosed in our study at 58.33 percent (El-Shenawy *et al.*, 2017) . The diagnostic rate in our study was lower than that reported by other researchers, as it was 30% for the number of samples, 12 out of 40 samples, while the researchers' rate was 51% for the number of samples, 92 out of 180 (Al-Sabary *et al.*, 2017) . The researchers also found a virulence factor gene, *cagA* at 70.6%, and this rate was lower than what was diagnosed in our study, where it was diagnosed at 100% (Al-Sabary *et*

al., 2017) . Other researchers in a study similar to our study where they detect a virulence factor gene *cagA* at 61 .2 %, and this percentage is lower than what was diagnosed in our study (El Khadir *et al.*, 2017) . In a similar study, 31% of bacteria were diagnosed, which is identical to the current study , and *cagA* was diagnosed with 77.27%, which is less than the percentage that was diagnosed in our study 100% (Pandya *et al.*, 2017) . In Muthanna Governorate, a study was conducted on the diagnosis of bacteria in terms of the presence of a number of virulence factors, including *ureaA*. The diagnosis rate was 39.28%, while in our study the percentage was higher, reaching 100% (Jabbar & AL-Obaidi, 2015) . Also, some researchers diagnosed the virulence factors genes *vacS1* and *s2* at 69.5 percent and 30.5%, respectively, and these percentages are lower than what was diagnosed with us, reaching 100 percent (Tiwari *et al.*, 2007) . In the province of Babylon, a study was conducted by a group of researchers detect a virulence factor gene, *urease A* at 73.9 %, and this percentage is somewhat similar to what was diagnosed in our study, which amounted to 100% (Al-Sabary *et al.*, 2017) . The reason for the difference in the percentages of diagnosis between our study and other studies that mentioned the small size of the samples or sampling from places where bacteria are abundant is likely the reason for the difference. or due to the small number of patients included in the study.

Conclusion

The results of current study confirm that it is possible to diagnosis *H. pylori* by amplifying 16S rRNA gene and *virulence genes ;cagA ,VacAs1/s2* , *IceA1* and *UreaA* by PCR, where this technique has accurate results in terms of specificity and sensitivity.

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