How to Cite:
Radhi, M. H., & Mohammed, G. J. (2022). Genotyping and virulence genes of Helicobacter pylori isolated from Patients with Gastroenteritis in Al-Diwaniyah Province/Iraq. *International Journal of Health Sciences*, *6*(S1), 11963–11974. https://doi.org/10.53730/ijhs.v6nS1.8028

Genotyping and virulence genes of *Helicobacter pylori* isolated from Patients with Gastroenteritis in Al-Diwaniyah Province/Iraq

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Abstract---The frequency distribution of *Helicobacter pylori* approximately 20 and 40% in developed nations and about 90%in developing countries. Diseases related to *Helicobacter pylori* is detected by a number virulence factors. So, the aim of this work is to detect virulence gene; *VACAm1* (Vaculating Cytotoxin Type Am1), *VACAm2* (Vacuolating Cytotoxin Type Am2), and *IceA2* (Induced by Contact with Epithelium Gene A2) in *Helicobacter pylori* isolates from patients with gastroenteritis symptoms using the molecular method (PCR). This study was conducted in Al-Diwaniyah Governorate / Iraq, and it included 40 patients, their ages ranged between 11 and 56 years, of both sexes. The samples were collected from October 2021 to February 2022. It depended on the diagnosis of the specialist doctor in diagnosing patients with gastroenteritis. Samples were collected from the Gastrointestinal Center at Al-Diwaniyah Teaching Hospital and the Internal Medicine Consultation, 20 stool samples were collected and placed in a sterile container and preserved with a phosphate buffer saline solution and 20 Tissue biopsy samples were taken from the patients' stomachs where biopsies excised from the gastric antrum using endoscopic forceps and preserved with normal saline solution. All samples (stool and biopsy) were kept deep frozen at -70°C in a freezer (Augelantoni/Italy) in the sub-blood bank of Al-Diwaniyah Teaching Hospital until examination by PCR. *Helicobacter pylori* were diagnosed according to the diagnostic gene 16SrRNA in 12 samples of tissue biopsy only with a percentage of 30%. The diagnosis of these positive samples was confirmed by a number of virulence factors: *vacAm1*, *vacAm2* and *IceA2*, with percentages of (8.33%, 100%, 66.66%, respectively). In addition, *Helicobacter pylori* isolates were fingerprinting by ERIC PCR (Enterobacterial repetitive intergenic consensus polymerase chain reaction) and according to it six ERIC (Enterobacterial repetitive intergenic consensus) types grouped into

International Journal of Health Sciences ISSN 2550-6978 E-ISSN 2550-696X © 2022.
Manuscript submitted: 27 March 2022, Manuscript revised: 9 April 2022, Accepted for publication: 18 May 2022

six clusters with 12 polymorphic variants were distinguished. In conclusion, the results detect the effectiveness of PCR in diagnosis of *Helicobacter pylori* based on housekeeping gene and virulence genes as well ERIC-PCR (Enterobacterial repetitive intergenic consensus polymerase chain reaction) analysis as a promising tool for molecular typing of *Helicobacter pylori* isolates and other bacteria.

***Keywords*---**Helicobcter pylori, VACAm1, VACAm2, Ice A2, ERIC PCR.

Introduction

Helicobacter pylori(H.pylori), Formerly known as *Campylobacter pylori*, is a gram-negative, microaerophilic, helical-shaped bacteria found in the stomachs of half the world's population. *H. pylori* is spread between humans and is usually acquired before the age of five, infected people have chronic gastritis, which can be asymptomatic for the rest of their lives in 85 percent of cases or progress to a variety of illnesses like peptic ulcers or gastric adenocarcinoma, which kills over 800,000 people worldwide each year (Robinson & Atherton, 2021) . *H. pylori* is the most well-known cause of stomach ulcers, duodenal ulcers, gastritis, and gastric cancer (Enzo et al., 2015) . It is associated with various stomach cancers, such as adenocarcinoma and mucosa-associated lymphoid tissue carcinoma (MALT) (Roesler et al., 2014). The combination of *H. pylori* virulence factors with the genetic information of host gastric epithelial cells has resulted in the development of gastric cancer (Oluwasola, 2014). *H. pylori* migrates toward the host gastric epithelium through flagella-mediated motility. Following that, bacterial adhesins bind to receptors on the host stomach epithelium. The displacement of bacteria from the stomach hinders peristalsis and gastric emptying. The release of toxins such as cag pathogenicity protein (cagA) and vacuolating cytotoxic protein (*vacA*) causes harm to the host tissue (Kao et al., 2016). The bacteria adapt to human colonization by producing virulence factors like urease, flagellin, catalase, cytotoxin, and lipopolysaccharide (LPS), which directly interfere with host cell functions and survive in the stomach for the rest of one's life, causing disease if not treated, and protecting the pathogen from clearance mechanisms like liquid flow, peristaltic movements, or mucous layer shedding(Parreira et al., 2013; Smolka & Backert, 2012) . 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) . To diagnose *H. pylori* infection, a variety of procedures are utilized, which are classified as invasive or non-invasive. The rapid urease test (RUT), microbiological culture, and biopsy-based polymerase chain reaction are all invasive methods (Sabbagh et al., 2019). Non-invasive approaches well documented include stool antigen testing (SAT), urea breath tests (UBT), and serological investigations(Stefano et al., 2018). *H. pylori* can also be detected using novel DNA-based approaches. The polymerase chain reaction (PCR) is a widely used method that has a sensitivity of up to 95% and a specificity of up to 95%. Because this type of identification may be performed in bioptates as well as

stool samples, PCR-based detection of *H. pylori* can be classified as either an intrusive or non-invasive approach, depending on the material used. Importantly, DNA-based approaches may identify not only active *H. pylori*, but also coccoid forms that might arise from stress, such as antibiotic use, rendering urease-based methods worthless (He et al., 2002; Momtaz et al., 2012; Sabbagh et al., 2019). The 16S rRNA gene is the most prevalent housekeeping genetic marker in *Helicobacter pylori*, and the amplification technique of the 16SrRNA gene has been demonstrated to be beneficial for the identification and phylogeny of the bacterium (Idris et al., 2020). Enterobacterial repetitive intergenic consensus (ERIC) is one of the repeating regions in the bacterial genome whose pattern and quantity vary. The enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) methodology is a rapid, dependable, and cost-effective method for molecular typing that detects genetic variability across strains (Mehr et al., 2017; Seifi et al., 2016) . This study aimed to detect some virulence genes of *H.pylori* isolates from patients with Gastroenteritis symptoms and fingerprinting the isolates using the molecular method ERIC-PCR (enterobacterial repetitive intergenic consensus polymerase chain reaction) .

Materials and Methods

Patients: This study was conducted in the Al-Diwaniyah teaching hospital in Al-Diwaniyah Governorate / Iraq and included 40 patients suffering from gastroenteritis symptoms whose ages ranged between 11 and 56 years from both sexes. The samples were collected from October 2021 to February 2022. The diagnosis of patients depending on the specialist doctor.

Samples collection : Samples were collected from the Gastrointestinal Center at Al-Diwaniyah Teaching Hospital and the Internal Medicine Consultation. 20 stool samples were collected and placed in a sterile container and preserved with a phosphate buffer saline solution. Tissue biopsy samples were taken from the patients' stomachs by excising from the gastric antrum using endoscopic forceps and preserved with normal saline solution. The number of biopsies was 20 samples. All samples (stool and biopsy) were kept deep frozen at -70°C in a freezer (Augelantoni/Italy) in the sub-blood bank of Al-Diwaniyah Teaching Hospital.

Detection of *H. pylori* by molecular methods

DNA extraction: The G-spin TM Total DNA Extraction Kit (iNtRON/Korea) and Presto™ Stool DNA Extraction Kit (Geneaid/USA) were used for biopsy and fecal DNA extraction respectively. Extraction was done according to the manufacturer's instructions. DNA quantity and quality were measured by a NanoDrop device (Thermo/USA).

Amplification of 16SrRNA gene and virulence genes by PCR

The GoTaq® Green PCR master mix kit (Promega / USA) was used to prepare the PCR master mix reactions for all genes, and according to the manufacturer's instructions this master mix was made. The total volume of the reaction was 25 µl comprising 5 µl of template DNA (5-50 ng), 2 µl of forward primer (10 µl), 2 µl of primer (10 µl), 12.5 µl of GoTaq® Green PCR master, and 3.5 µl of PCR water.

The primer was designed based on the references (Table 1). The PCR heat cycler conditions protocol for each gene was calculated using the online Optimase Protocol Writer™ app and done by using conventional PCR thermocycler. performed using the Touchdown protocol for the *16SrRNA*, *IceA2*, and *ERIC* genes (Table 2) and a conventional PCR for the *vac A m1* and *vac AM2* genes (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)
vacAm1	F: GGTCAAAATGCGGTCATGG R: CCATTGGTACCTGTAGAAAC	290	(Idowu et al., 2019)
vacAm2	F: CATAACTAGCGCCTTGCAC R: GGAGCCCCAGGAAACATTG	352	
IceA2	F:GTTGTCGTTGTTTTAATGAA R:GTCTTAAACCCCACGATTAAA	120	(Essawi et al., 2013)
ERIC	F:ATGTAAGCTCCTGGGGATTCAC R:AAGTAAGTGACTGGGGTGAGCG		(Finger et al., 2006)

Table (2)
Touchdown protocol of *IceA2* gene

Touch Down PCR step	Temp.	Time	repeat
Initial Denaturation	95°C	2min.	1
Denaturation	95 °C	30sec.	14 cycle
Annealing	55.4 °C decrease 0.5 per cycle	30sec	
Extension	72 °C	20sec	
Denaturation	95 °C	30sec.	19 cycle
Annealing	48.4 °C	30sec	
Extension	72 °C	20sec	
Final extension	72 °C	5min.	1
Hold	4 °C	Forever	-

Touchdown protocol for amplification of *16SrRNA* and *ERIC* gene was same as *IceA2* gene with some changes in some steps, which are for (*16srRNA* :Annealing step 57.1 °C , 50.1 °C) , (*ERIC*: Annealing step 64.9°C , 57.9°C).

Table (3)
PCR Thermocycler conditions of *vacAm1* gene

PCR step	Temp.	Time	Repeat
Initial Denaturation	95°C	2 min	1

Denaturation	95°C	30sec.	29 cycle
Annealing	56°C	30sec	
Extension	72°C	30 sec	
Final Extension	72°C	5min	1
Hold	4°C	Forever	-

PCR Thermocycler conditions of *vacAm2* gene was the same of *vacAm1* gene with some changes in annealing step which is (57.8°C).

PCR product analysis

For analysis of PCR products agarose gel electrophoresis was used, 1.5% agarose gel was used, including 0.5 µg/ml ethidium bromide, and the DNA bands were separated by electrophoresis and visualized by the UV Transilluminator (Wisd/Korea).

Results

Identification of *H.pylori* by biochemical and molecular assays

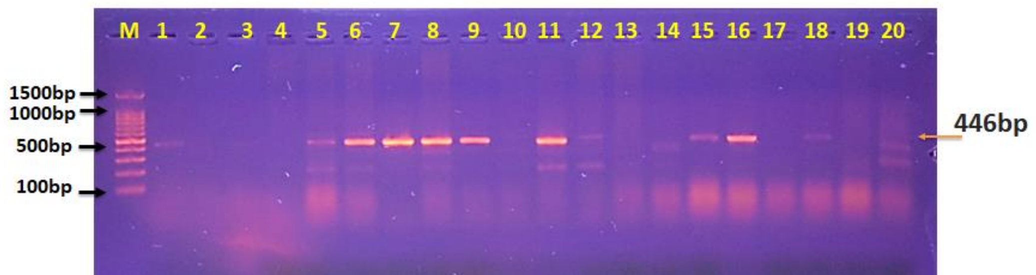
H. pylori was diagnosed primarily by the urease test, which is one of the traditional biochemical tests used to identify *H. pylori* (MacFaddin, 2000). *H. pylori* antigen test kit (high top /China) was used to diagnose stool samples, and all 20 samples showed positive results.

The bacteria were diagnosed using polymerase chain reaction (PCR) technology after DNA extraction from biopsy and stool samples and DNA amplification, and the diagnosis was made by the *16SrRNA* gene, 12 out of 40 samples were diagnosed as *H. pylori* which were from biopsy tissues only and were at a rate of (30%) as mentioned in Table (4).

Detection of virulence factors and *ERIC* genes: Positive samples that were diagnosed by the *16SrRNA* gene were used to detect some genes of virulence factors and *ERIC* gene that appeared in different proportions as mentioned in Table (4) and Figures (1) to (4).

Table (4)
The rate (%) of *16SrRNA* gene, virulence factors and *ERIC* genes in *H.pylori* isolates

Genes	Positive samples	%
16SrRNA	12	30
VacAm1	1	8.33
vacAm2	12	100
IceA2	8	66.66
ERIC	12	100



Figure(1): Agarose gel electrophoresis image that showed PCR product analysis for detection 16SrRNA gene of *H. pylori* in biopsy patients samples. M (Marker ladder 1500-100bp). Lane (1-20) Showed some positive 16Sr RNA gene of *H. pylori* at 446bp product size.

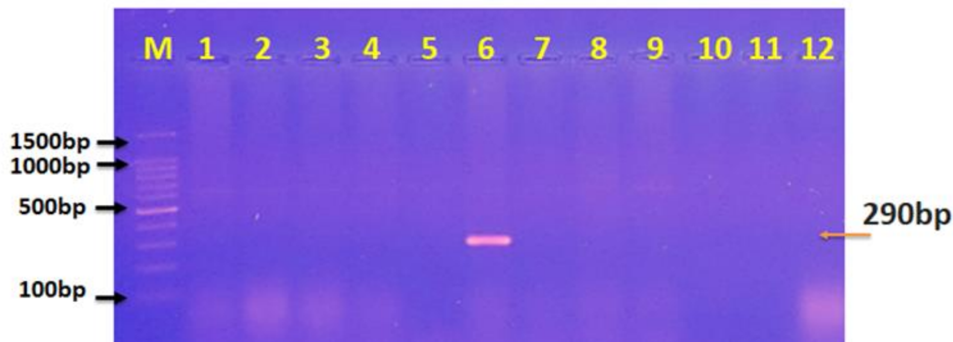


Figure (2): Agarose gel electrophoresis image that showed PCR product analysis for detection *vacAm1* gene of *H. pylori* in biopsy patients samples. M (Marker ladder 1500-100bp). Lane (1-12) No. of isolates that showed some positive *vacAm1* gene at 290bp product size.

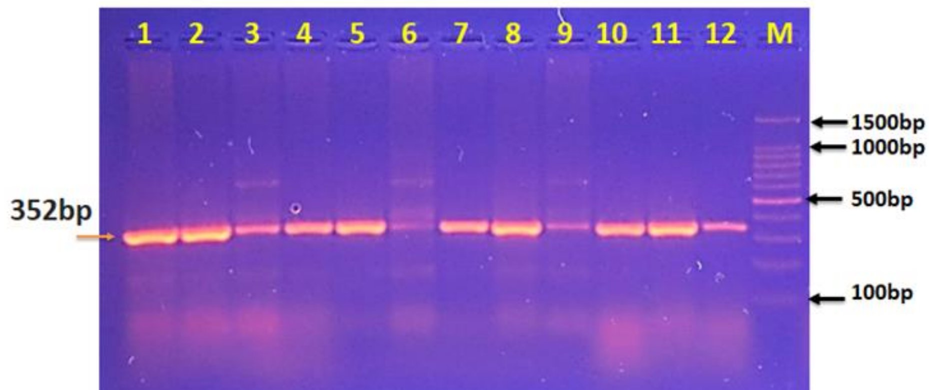


Figure (3): Agarose gel electrophoresis image that showed PCR product analysis for detection *vacAm2* gene of *H. pylori* in biopsy patients samples. M (Marker ladder 1500-100bp). Lane (1-12) No. of isolates that showed some positive *vacAm2* gene at 352bp product size.

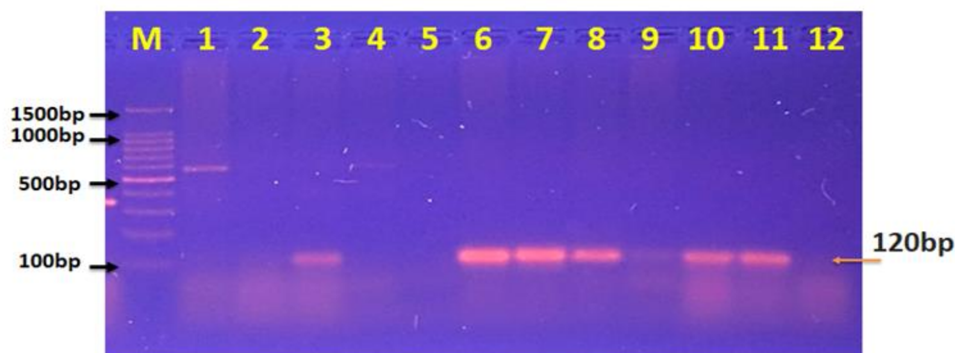


Figure (4): Agarose gel electrophoresis image that showed PCR product analysis for detection *IceA2* gene of *H. pylori* in biopsy patients samples isolates. M (Marker ladder 1500-100bp). Lane (1-12) No. of isolates that showed some positive *IceA2* gene at 120bp product size.

Fingerprinting by ERIC-PCR

The enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) profiles permitted classified the 12 isolates into 6 genotypes ,that were showed into six clusters with 12 polymorphic variants that were classified as follows : Genotype I had two polymorphic variants, Genotype II had one, Genotype III had three polymorphic variants, Genotype IV had one, Genotype V had one, and Genotype VI had three polymorphic variants, The result is shown in table (5) and figure (5).

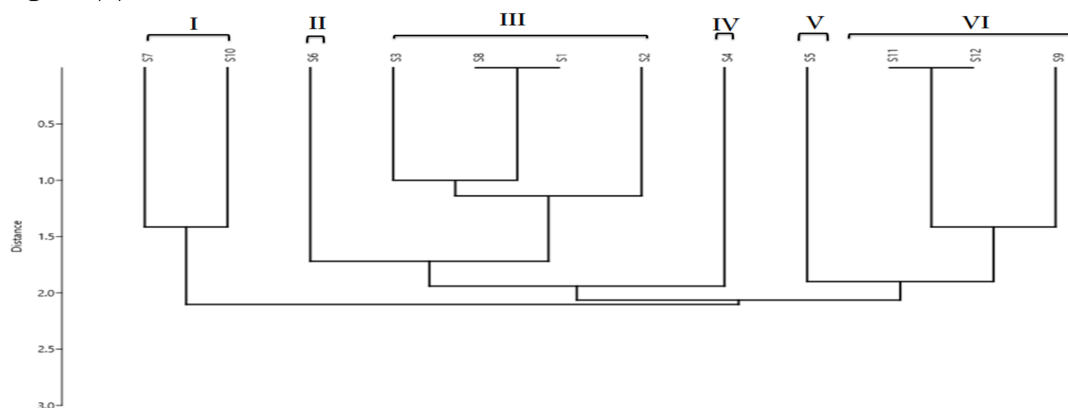


Figure (5): RAPD-PCR dendrogram tree analysis of *H. pylori* isolates by using (Paleontological Statistics version 4.0). The Cluster analysis using (algorithm Ward's method) were showed 6 genotypes variants within 10 polymorphic variants between 12 *H. pylori* clinical isolates.

Table (5)
RAPD-PCR Cluster analysis and polymorphic variants for 12 *H. pylori* isolates

Cluster No.	Isolate No.	No. polymorphic variants
Genotype I	S7, S10	2
Genotype II	S6	1
Genotype III	S1, S2, S3, S8	3
Genotype IV	S4	1
Genotype V	S5	1
Genotype VI	S9, S11, S12	3
Total:6	12	10

Discussion

Our study included the diagnosis of *H. pylori* bacteria from targeted patients who suffer from gastro-intestinal diseases. Stool samples and tissue biopsies were taken from patients. The *16SrRNA* gene is the most prevalent housekeeping genetic marker in *Helicobacter pylori*, and the amplification technique of the *16S rRNA* gene has been demonstrated to be beneficial for the identification and phylogeny of the bacterium (Idris et al., 2020) . The urease test was used to diagnose tissue biopsies samples and then it was confirmed by the diagnostic gene *16SrRNA*, after which the positive samples were screened for the detection of some virulence factor genes; *vacAm1*, *vacAm2* and *IceA2*. In a study similar to ours (Szymczak et al., 2020), which included diagnosing bacteria by PCR, 50% of the samples were positive based on the *16srRNA* gene in 20 samples out of 40, and this percentage was higher than what was diagnosed in our study, where the percentage was 30%. Researchers in one of the studies (Idris et al., 2020) found a diagnosis rate of bacterial infection at 37.33%, which is close to our diagnosed rate. Bacteria were diagnosed based on the *16srRNA* gene at a rate of 7.65% in (Al-Jumaili et al., 2021) study, and this percentage was higher than the percentage diagnosed in our study. In Sudan,a study was conducted by (Mamoun et al., 2015) to diagnose *Helicobacter pylori* in a number of patients with biopsy samples, and by 67% for 12 out of 18 samples, while it was diagnosed by 30% for 12 out of 40 samples. In a study in South Africa (Idowu et al., 2019) after diagnosing bacteria from patients with gastritis based on the *16 srRNA* gene, it was confirmed by the virulence factors *vacAm1*and *vacAm2* that diagnosed by (69.2% and 21.4% respectively) .While it was diagnosed in our study 8.33 % and 66.66 % respectively. Also, in another study in South Africa (Tanih et al., 2010), researchers were able to diagnose bacteria and confirm their virulence factors *vacAm1*, *vacAm2*, *IceA2*, in the following percentages (8%, 50%, and 58%, respectively). The researchers agreed with us in diagnosing *vacAm1* at a very close rate, while they differed with our study in diagnosing *vacAm2*, where our percentage was higher. As for the *IceA2* gene, in our study had a slightly higher rate, where our diagnostic rates reached (8.33%, 100%, and 66.66%, respectively). In a Tunisian study by (Ben Mansour et al., 2010), a number of researchers were able to diagnose bacteria based on factors of the same virulence genes used in our study, and the diagnosis rate for samples positive for the virulence factors *vacAm1*, *vacAm2*, and *IceA2* was 30%, 58%, and 16%, respectively), and these percentages were lower than that reported in our study, except for *vacAm1*, where the percentage was lower still. In a study by (Caner et

al., 2007), they agreed with us in diagnosing bacteria based on the virulence factor *IceA2*, where the percentage was close to 66.6%, but in the virulence factor *vacAm2*, they differed with us and the percentage was 68.7% less than what was diagnosed in our study. In a Jordanian study by (Nimri et al., 2006),researchers were able to diagnose virulence factors in *H. pylori* in varying proportions to the rates diagnosed in our study that were (48.9%, 51%, and 73.6%) for virulence factors *vacAm1*, *vacAm2*, and *IceA2*, respectively. Regarding the results of *H.pylori* genotyping by ERIC PCR ,They were somewhat differ from what reported in a study in Mexico, researchers (González-Vázquez et al., 2012) identified a number of strains of *H. pylori* bacteria, which can be classified according to genetic diversity into 39 genetic variants, while in our study, 12 genetic variants; the reason for this difference in the number of genetic variants may be due to decrease in the number of isolates obtained in our study . Also, in a study by (Hussain et al., 2004) that included a number of different geographical regions and a number of countries, a number of strains of *H. pylori* bacteria were reported, which can be classified according to genetic diversity into 22 genetic variants in the European African cluster and 23 genetic variants in the Irish, East-Asain, and Amarindish clusters, while our study showed 12 genetic variants, where we noticed a comparable ratio, and this difference is due to the large number of samples from these geographical areas, while it showed 15 genetic variants in the European East Asian cluster, and this ratio is close to the number of variants that were reported in our study. The genetic variation in each clusters in these study confirms that strains obtained from various clinical samples have polymorphic genetic background and have different virulence profile.Currently, fingerprints created by ERIC-PCR proposed the existence of such repetitive sequences in H. pylori (Ann-Catrin *et al.*,2000).

Conclusion

The results of the current study confirmed that it is possible to diagnose *H. pylori* by amplifying the *16SrRNA* gene, virulence genes such as *vacAm1*, *vacAm2*, *IceA2* using PCR, where this technique having accurate results in terms of specificity and sensitivity, and the genotyping by using repetitive PCR such as ERIC-PCR analysis as a promising tool for molecular typing of *H.pylori* isolates and other bacteria.

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