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Isolation and Diagnosis of the Bacteria Causing Gastric Ulcers and Identification of the Causative Gene using PCR Technique

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A B S T R A C T

Gastric ulcers represent a significant global health burden, affecting millions of individuals annually. *Helicobacter pylori* is an important pathogen in humans and is considered the main cause of refractory gastritis. It is also associated with the development of peptic ulcers, atrophic gastritis, and gastric tumors. This study aimed to detect the presence of *H. pylori* in stool samples from patients with gastric ulcers using polymerase chain reaction (PCR) and targeting the cagA, vacA, and ureC genes. Stool samples were collected from 200 symptomatic patients and subjected to culture analysis to isolate *H. pylori*. The presence of *H. pylori* isolates was confirmed by culture analysis, Gram staining, biochemical assays, and PCR tests targeting the cagA, vacA, and ureC genes. Of the 200 stool samples, 125 showed positive results for *H. pylori* colonies in males and 75 in females on agar plates., which were confirmed by Gram staining and biochemical assays. Genomic DNA of *Helicobacter pylori* was taken based on the cetyl trimethyl ammonium bromide protocol (CTAB). Nested PCR was performed using specific primers (HP-F and HP-R), (ccagA-F and cagA-R), (vacAs1 *and vacAs2), (vacAm1 and vacAm2), and (VacAF* and *VacA*R) sets for the ureC gene, as well as the virulence genes of cagA and vacA. Gel electrophoresis analysis was performed to visualize the PCR products. PCR results showed the presence of cagA, vacA, and ureC genes in *H. pylori* isolates. These results indicate the prevalence of *H. pylori* infection and the presence of virulence genes associated with peptic ulcer disease in the study population.

Introduction

Gastric ulcers represent a significant global health burden, affecting millions of individuals annually (Jaiswal, Falguni *et al.,* 2021; Chan and Lanas, 2017; King *et al.,* 2022). Ulcers are painful sores that can occur in the mucosa or skin and are characterized by the sloughing of inflamed, soft, dead tissue (Wong *et al.,* 2004; Burlando *et al.,* 2021; Stark *et al.,* 2010; Cozzani *et al.,* 1998). Among the various factors contributing to the development of gastric ulcers, infection with *H. pylori* has been identified as a primary causative agent (OmarEl-, Amieva, 2008; van Amsterdam *et al.,* 2006). Gastric ulcers are a disruption in the protective mucosal barrier of the stomach lining that ruptures through the muscular mucosa, and these ulcers have a diameter

exceeding 5 mm and display visible depth (Radin *et al.,* 2015; Kim *et al.,* 2020; Blanchard and Czinn, 2011). This Gram-negative bacterium colonizes the gastric mucosa, triggering inflammatory responses and leading to tissue damage, ultimately culminating in the formation of ulcers (Sonis, 2012; Montassier *et al.,* 2014). It has been observed that approximately 80% of patients with gastric ulcers are infected with *H. pneumonia* (Kuipers *et al.,* 1995). *H. pylori* is a Gram-negative, spiral-shaped bacterium that is resistant to acid and colonizes in the luminal surfaces of the gastric epithelium (Brigitte *et al.,* 2018). Understanding the precise etiology of gastric ulcers is paramount for effective diagnosis and treatment (Marshall *et al.,* 1983; Wilson *et al.,* 2010). Traditional diagnostic methods for *H. pylori* infection, such as culture and histological examination, are labor-intensive, time-consuming, and may lack sensitivity (Hooi *et al.,* 2017; Sahebkar *et al.,* 2017). It is well known that infection attacks in humans impact more than half of the people. The dispersal of infections by *H. pylori* varies greatly depending on socioeconomic and hygienic conditions, with higher rates found in developing pastoral places (over 79%) in contrast to civilly developed places (almost 40%). infection by *H. pylori* is often gained within childhood, and while many infected individuals have not any symptoms, about 30% might have mild to sharp gastrointestinal disorders in the upper digestive tract, comprising stomach inflammation, peptic ulcer, and cancer in gastric or mucosa-assisted lymphoid tissue (Gaiani *et al.,* 2018). Helicobacter is a curved bacillus that belongs to the Gram-negative bacteria. It thrives in environments with moderate levels of oxygen and possesses a lipid structure that protects its 4 to 8 polar flagella. Helicobacter bacteria are known to produce urease, catalase, and cytochrome oxidase enzymes (Naranjo *et al.,* 2012). The presence of urease is particularly significant in *H. pylori,* as it enhances the bacteria's virulence by converting urea into ammonia and water, thereby raising the pH and neutralizing the surrounding acidic environment.

Additionally, Helicobacter utilizes other factors such as lipase, adhesions, catalase, and platelet activator factor to contribute to its overall pathogenicity (Blanchard and Nedrud, 2012). Various diagnostic methods are utilized to diagnose the infection by *H. pylori*. Some of them are direct methods such as histopathology, chemistry of the immune system (IHC), RUT, or Campylobacter-like organism test. In addition to cultivation, these methods provide valuable genotype data and resistance data for antibiotics. While indirect methods such as the *H. pylori* breath test and stool antigen test (SAT) have been preferred in determining efficient infection. Tests for antibodies, particularly serology, are present widely and highly precise but lack strain determination (Sasatsu *et al.,* 2013). Where *H. pylori* has specific genes or virulence factors has a big role in gastric ulcer disease pathogenesis such as cytotoxin-associated gene A (Cag A) and vacuolating cytotoxin A (Vac A), that are motivated by interaction with epithelial membrane antigen. Vac A toxin, which forms pores, not only induces vacuole formation only in the stomach epithelial layer and cells of the inner wall but also aids in the colonization of *H. pylori* in the stomach (Ahmed and Monjur, 2019). As such, there has been a growing emphasis on molecular techniques, particularly polymerase chain reaction (PCR), for rapid and accurate detection of *H. pylori* and its virulence factors. PCR offers several advantages over conventional methods, including high sensitivity, specificity, and the ability to detect low concentrations of target DNA (Shi *et al.,* 2023; Sokmensuer *et al.,* 2018). Moreover, PCR enables the identification of specific genes associated with bacterial virulence, shedding light on the pathogenic mechanisms underlying gastric ulceration. In this context, this study focuses on the isolation, diagnosis, and genetic identification of bacteria causing gastric ulcers, with a particular emphasis on *H. pylori*, using the PCR technique (Orhan *et al.,* 2018; Yao *et al.,* 2023). Recently, PCR targeting *H. pylori*-specific genes has emerged as an alternative diagnostic approach, often utilizing DNA obtained from stomach biopsies or gastric secretion and stools.

Stool specimens are a complex combination of bacteria and chemicals, including substances that can inhibit the PCR analysis; however, there are straightforward techniques to effectively extract DNA from human stools. Nested-PCR, which focuses on 23S rRNA factor, has demonstrated excellent sensitivity in detecting *H. pylori.* By isolating and characterizing the causative bacteria, we aim to enhance our understanding of the microbial etiology of gastric ulcers and pave the way for targeted therapeutic interventions (Vaez *et al.,* 2017). By isolating and characterizing the causative bacteria, we aim to enhance our understanding of the microbial etiology of gastric ulcers and pave the way for targeted therapeutic interventions (Gong *et al.,* 2011). Additionally, the identification of specific virulence genes using PCR holds promise for the development of novel diagnostic tools and therapeutic strategies tailored to individual patients based on their bacterial genotype

(Lange *et al.,* 1996; Yeh *et al.,* 2018; Censini *et al.,* 1995; Ikenoue *et al.,* 2001). *H. pylori* has specific genes or virulence factors that have a big role in gastric ulcer disease pathogenesis such as cytotoxin-associated gene A (Cag A) and vacuolating cytotoxin A (Vac A) that are motivated by interaction with epithelial membrane antigen. Upon reaching the stomach, *H. pylori* utilizes its urease in order to neutralize the acidic medium in the stomach; after that, it adheres to epithelial cell receptors on the stomach wall using its cohesion molecule. Cag A, a high immune protein, influences maliciousness by promoting IL-8 production and then activating factor kB in the nucleus. Similarly, ice A raises mucosal IL-8 coding (Mitsuno *et al.,* 2001; Abu-Taleb *et al.,* 2018; Till *et al.,* 1996; Aghdaei *et al.,* 2018). This cascade triggers the activation of the gastric epithelial layer's innate immune response and neutrophils, which cause stomach ulcer formation.

Vac A toxin, which forms pores, not only induces vacuole formation only in the stomach epithelial layer and cells of the inner wall but also aids in the colonization of *H. pylori* in the stomach (Baghaei *et al.,* 2017; Bravo *et al.,* 2012; Ilver *et al.,* 1998; Namwat *et al.,* 2008). This research endeavors to contribute to the advancement of precision medicine in the management of gastric ulcers, ultimately improving patient outcomes and reducing the burden of this debilitating condition; thus, this article aims to detect the existence of *H. pylori* in specimens that are taken from infected persons using PCR through targeting *cagA*, *vacA*, *iceA1*, and *iceA2* virulence genes.

Materials and Methods

Research community

This study was conducted at Kirkuk General Hospital in Iraq from 1 January 2023 to 1 June 2023. 200 samples were collected from patients suffering from symptoms similar to those of stomach ulcers, such as indigestion, heartburn, weight loss, nausea, and loss of appetite.

Sampling

Stool samples were collected from a total of 200 patients, including 125 males and 75 females, who requested medical consultation at the aforementioned hospital. These patients showed symptoms similar to those of stomach ulcers, such as indigestion, heartburn, weight loss, nausea, and loss of appetite.

The Cultural Analysis

Stool samples were stored at 4°C. They were then diluted to a 20% suspension in phosphate-buffered saline (PBS) and filtered using a 250-μm strainer to remove large particles. A 200-ml aliquot of the suspension was placed on plates containing tryptic soy agar plus sheep blood (5% sheep blood); blood was added to the medium to obtain the nutrients needed for microbe growth and allow hemolysis and glucose release of essential carbohydrates for some bacteria (Guaman, Jhoan *et al.,* 2018). The Petri dishes were incubated at 37°C for 72 h, and the plates were exposed to a microaerosol containing 5% O2, 10% CO2, and 85% N2. To confirm the presence of *H. pylori* isolates, additional tests were performed, including Gram staining and biochemical assays to determine catalase, oxidase, and urease production. Furthermore, a PCR test was performed to confirm the presence of *H. pylori* (Fraser *et al.,* 1994; Wilson, 1987).

Extraction of the genomic DNA of the *H. pylori*

Bacterial genomes were obtained using the CTAB protocol (Sambrook, Joseph, 2001). First, a loop filled with bacteria was mixed with distilled water (about 1.5 ml) and then transferred to the centrifuge well at 1000 g for 10 min. The supernatant was removed, and a mixture of 270 l of T/E buffer, 30 ml of 10% SDS, and 5 ml of proteinase K was added to an Eppendorf tube, which was then incubated overnight at 50 °C. Adjacent to the incubation period, 100 liters of 5M NaCl were introduced into the tube along with 80 liters of CTAB/NaCl solutions that had been rewarmed in a 65°C water bath, followed by vortexing and incubation at 60°C for 12 min.

A solution of chloroform-isoamyl alcohol (24:1) (200 mL) was placed in the tube and mixed well in the vortex. The suspension was centrifuged at 10,000 g for 15 minutes at a temperature of 10 °C, and the liquid was frozen. They were collected and transferred to another tube, in which isopropanol was added to precipitate the DNA.

The tube was incubated at -20 °C for 32 min, and finally, it was centrifuged at 12,000 g for 10 min. The excess was shaken off, and the pellet was resuspended in a tube containing 1 ml of chilled 70% ethanol and then centrifuged at 12,000 g for an additional 5 min at 10 °C. The excess was shaken off by air drying, then the DNA pellet was dissolved in 50 l of T/E buffer, incubated for 30 minutes at 37 °C, and finally stored at 4 °C overnight.

The BP of the DNA was then detected by the gel electroforces. Image (5) gel electrophoresis of PCR amplification for *H. pyloir* DNA depending on vacAF and vacAR primers. Lanes: M, 1000 bp DNA lader; lane 3 is negative; lanes 1, 2, 4,5 are positive with amplification of size 352 bp.

Diagnosis of cagA, ureC and mosaic vacA virulence genes

In the present research, PCR was used to confirm the presence of *H. pylori* isolates by targeting the ureC gene and also to detect the presence of vacA and cagA genes that have a critical role in the pathogenesis of gastric ulcer disease. Nested PCR reactions were performed using specific primer sets chosen from published studies shown in Table 1 (Atherton *et al.,* 1995; Harrison *et al.,* 2017).

PCR reaction conditions

PCR operations were performed in 50µL as a whole volume, which consisted of 10 mM Tris-HCl, 1.5 mM magnesium chloride, 0.2 mM of each DNTP, 25 nmol for individual primers, and 2.5 units of Taq polymerase.

PCR amplification conditions for each pair of primers comprised pre-incubation for 3 min at 94 °C, then 35 cycles of 30 s at 94 °C were done for the step of denaturation, 30 s at 58 °C for the step of annealing, 30 s at 72 °C for extension, and 10 min at 72 °C for ultimate extension. PCR yields have been visualized via gel electrophoresis by 1.5% agarose gels and the stain of ETBR. Figure 1 shows *H. pylori* isolates on blood agar.

Results and Discussion

Out of a total of 200 samples cultured on Tryptic soy agar plus sheep blood, 125 samples from males and 75 from females showed positive results by forming colonies on the medium. *H. pylori* colonies were identified based on their characteristic appearance, including being clear, round, with smooth edges and a convex shape, and ranging from 0.5 to 1.5 mm in diameter. The identification process included performing a positive urease test on Christensen's urea agar, a positive catalase test using H2O2, and observing the typical curved shape of the bacteria after staining with crystal violet. These characteristic features and tests confirmed the presence of *H. pylori* bacteria. In addition, Gram staining confirmed that these colonies belonged to *H. pylori.* The following gel electrophoresis images show the results of nested PCR tests using the specific primer sets listed in Table 1 of Five primers for PCR tests were performed after the culture step to isolate the virulence genes responsible for causing peptic ulcer disease. Figure (2) gel electrophoresis of PCRr amplification for *H. pylori* DNA depending on primers HP-Fm and HP-Rm. Lanes: M, 1000 bp DNA lader; lane 2 is negative; lanns 1, 3, 4, 5, 6 are positive with amplification of size is 294 bp. Figure (3): Gel electrophoresis of PCR amplification for *H. pylori* DNA depending on cagA-F and cagA-R primers. Lanes: M, 1000 bp DNA lader; lane 3 is a negative; lane 1, 2, 4, 5, 6 are positive with amplification length of size 352 bp. Figure 4: Gel electrophoresis of PCR amplification for *H. pylori* DNA depending on vacAs1 and vacAs2 primers. Lanes: M, 1000 bp DNA lader; 1, 2, 3, 4, 5, are positive with. amplification of size 259 bp. Figure 5: Gel electrophoresis of PCR amplification for *H. pylori* DNA depending on vacAm1 and *vacA*m2 primers. Lanes M, 1 kb bp DNA lader; lane 1, 3 are negative; lanes 2,4,5,6 are positive, showing amplification of size 290 bp. Figure 6: gel electrophoresis of PCR amplification for *H. pylori* DNA depending on vacAF and vacAR primers. Lanes: M, 1000 bp DNA lader; lane 3 is negative; lanes 1, 2, 4,5 are positive with amplification of size 352 bp.

This research included isolating and diagnosing the bacteria that cause stomach ulcers, specifically *H. pylori*, and identifying the genes that cause them using polymerase chain reaction technology. *H. pylori* was typically isolated and diagnosed during sample collection, which included stool samples from infected patients. *H. pylori* bacteria were cultured under specific conditions, such as a microaerobic environment with high humidity and temperatures around 37°C.

Specialized media such as tryptic soy agar supplemented with antibiotics were used to inhibit other bacteria and promote the growth of *H. pylori*. Isolates were identified by staining the samples and examining them microscopically to confirm the presence of *H. pylori*, as indicated by its characteristic curved shape. The samples were tested for urease activity, one of the main enzymatic properties of *H. pylori* bacteria. The method used is a polymerase chain reaction, which targets specific genes of *H. pylori* bacteria (such as ureC, vacA, or cagA) using PCR. The causative genes were identified using PCR because various *Helicobacter pylori* genes are associated with virulence and pathogenesis, such as vacA (vacuolar cytotoxin A-related gene) and ureC (urease subunit C).

Table.1 Primer pairs for *H pylori* amplification

primers	gene	Sequence of primers	Product length	References
HP-F **HP-R**	ureC	F:GGATAAGCTTTTAGGGGTGTTAGGGG R:GCTTACTTTCTAACACTAACGCG	294 bp	(Peek *et al.*, 1995)
cagA-F **cagA-R**	cagA	F:AGG GAT AAC AGG CAA GCT TTT GA R:CTG CAA AAG ATT GTT TGG CAG A	352 bp	(Zuo *et al.*, 2022)
***vacA*s1** ***vacA*s2**	vacA	F: ACTAATATTGGCACACTGGATTTG R: CTCGCTTGATTGGACAGATTG	259 bp	(Ogiwara *et al.*, (2009)
***vacA*m1** ***vacA*m2**		F: CAATCTGTCCAATCAAGCGAG R: GCGTCAAAATAATTCCAAGG	290 bp	(Ogiwara *et al.*, (2009)
***VacA*F** ***VacA*R**		F: ATGGAAATACAACAAACACAC R: CTGCTTGAATGCGCCAAAC	352 bp	(Santiago *et al.*, 2015)

Figure.1 *H pylori* isolates on blood agar

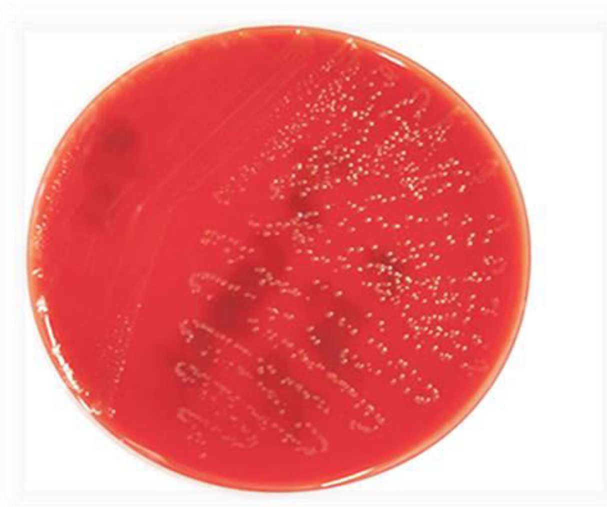
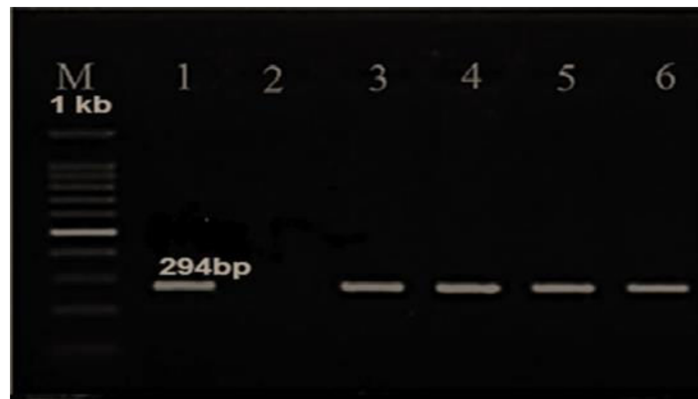
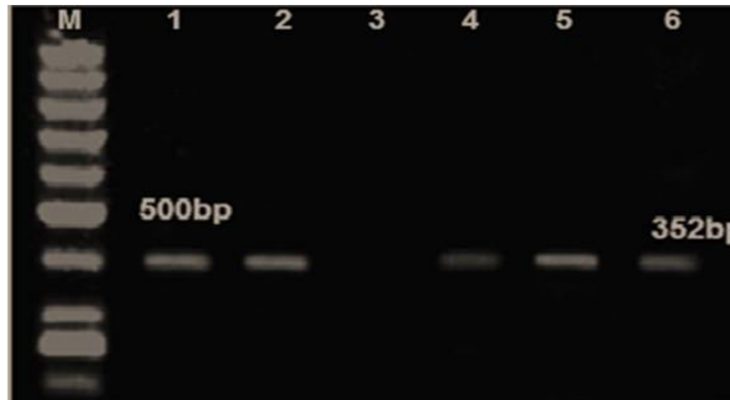


Figure.2 Gel electrophoresis of PCR amplification for H. pylori DNA depending on primers HP-Fm and HP-Rm.



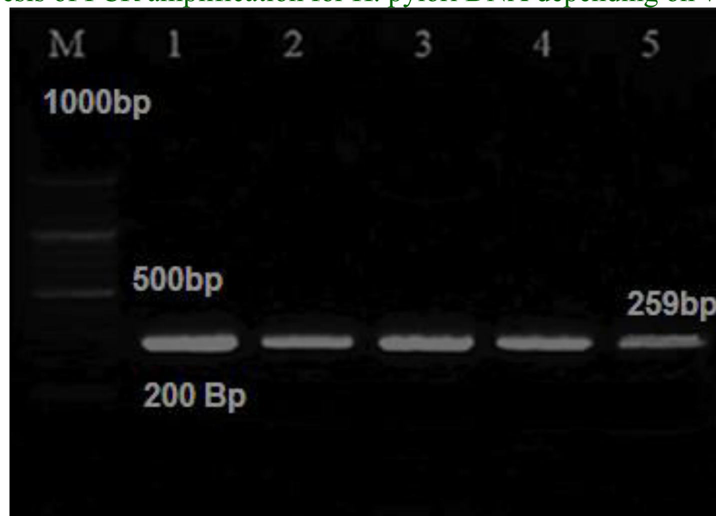
Lanes: M, 1000 bp DNA lader; lane 2 is negative; lanns 1, 3, 4, 5, 6 are positive with amplification of size is 294 bp.

Figure.3 Gel electrophoresis of PCR amplification for H. pylori DNA depending on cagA-F and cagA -R primers.



Lanes: M, 1000 bp DNA lader; lane 3 is a negative; lane 1, 2, 4, 5, 6 are positive with amplification length of size 352 bp.

Figure.4 Gel electrophoresis of PCR amplification for H. pylori DNA depending on vacAs1 and vacAs2 primers.

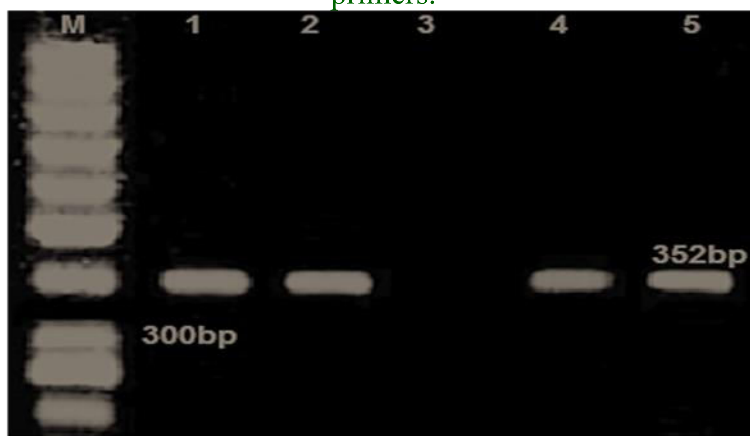


Lanes: M, 1000 bp DNA lader; 1, 2, 3, 4, 5, are positive with. amplification of size 259 bp.

Figure.5 Gel electrophoresis of PCR amplification for H. pylori DNA depending on vacAm1 and vacAm2 primers.

Lanes M, 1 kb bp DNA lader; lane 1, 3 are negative; lanes 2,4,5,6 are positive, showing amplification of size 290 bp.

Figure.6 Gel electrophoresis of PCR amplification for H. pylori DNA depending on vacAF and vacAR primers.



Lanes: M, 1000 bp DNA lader; lane 3 is negative; lanes 1, 2, 4,5 are positive with amplification of size 352 bp.

PCR primers were designed complementary to specific regions of target genes, ensuring specificity and sensitivity and ensuring the extraction of genomic DNA from cultures of *H. pylori* isolated from samples using a standard PCR protocol, which typically included denaturing, annealing, and extension steps optimized for the specific primers. The PCR products were then separated on an agarose gel to ensure that the target genes were amplified based on size. By sequencing the PCR products, the identity of the amplified genes is verified, which is necessary for epidemiological or research purposes. Identifying *H. pylori* and virulence genes based on PCR helps in accurate diagnosis and treatment planning for stomach ulcers and associated conditions. The genetic diversity and distribution of virulence genes among *H. pylori* strains contribute to epidemiological studies and the development of targeted therapies. The success of PCR depends on the quality and quantity of extracted DNA, which can be affected by sample collection and storage conditions. Optimization of PCR conditions and accurate interpretation of results are crucial to reducing false results. Ethical guidelines were also followed when conducting research involving human subjects and biological specimens.

Identifying the genes responsible for stomach ulcers using PCR technology and isolating and diagnosing the causative bacteria, especially *Helicobacter pylori*, constitutes a major advance in medicine and microbiology. It included isolating *Helicobacter pylori* bacteria after sampling, cultivating them under specific conditions, and using selective media to ensure accurate identification. Rapid urease tests, PCR-based methods, and histopathology are diagnostic techniques that further verify infection and measure virulence.

PCR is particularly sensitive and specific in identifying specific genes - such as vacA, cagA, and ureC - associated with *Helicobacter pylori* pathogenicity. This molecular technique supports the study of strain diversity, epidemiology and therapeutic approaches and helps in correct diagnosis.

However, difficulties including poor sample quality, the possibility of false results, and ethical considerations highlight the necessity of strict procedures and interpretations. Overall, PCR-based genetic identification, diagnosis, and isolation are expanding our knowledge of gastric ulcers and their treatment, opening the door to more focused treatments and better outcomes for individuals receiving care. Further developments in molecular methods should provide additional understanding of the complex relationships that exist between bacteria and their hosts, influencing future directions in microbiology and gastrointestinal pathology.

Author Contributions

Shler Ali Khorsheed: Investigation, formal analysis, writing—original draft.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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