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Detection of Helicobacter Pylori's Virulence Gene (*UreA*) and its Influence on the Result of Rapid Urease Test (RUT)

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ABSTRACT

UreA is an important virulence factor of Helicobacter pylori that, along with *UreB* and *UreC*, produces urease. Urease enzyme helps the bacterium to colonize the human stomach through metabolizing urea in order to neutralize the gastric environment. The current study aimed to detect the prevalence of the *H. pylori*'s *ureA* virulence factor gene, and to investigate the influence of this gene on the result of the rapid urease test (RUT). Eighty stomach biopsy samples were isolated from participants who were suspected to be infected with *H. pylori* in Erbil city. Participants were 36 males and 44 females, aged between 18 and 67 years. The results showed that 42 (52.5%) of the participants were positive for *H. pylori* when tested by RUT, while 59 (73.8%) of the patients showed positive *H. pylori* infection when tested by polymerase chain reaction (PCR) targeting the 16S rRNA gene. The results of the PCR test based on the *ureA* gene revealed that 42 (52.5%) of the samples were positive. The important finding of this research is the presence of 100% compatibility between positive samples of RUT and *ureA* genes. It can be concluded from this study that a person may be infected with *H. pylori*, but the RUT test fails to detect the infection if the bacteria lack the *ureA* gene, indicating a direct impact of this gene on the result of RUT, which is a defect of RUT.

KEYWORDS: *H. pylori*; RUT; *ureA* gene; *16S rRNA*; PCR.

الخلاصة

UreA هو عامل ضراوة مهم في Helicobacter pylori والذي مع UreB و UreC، ينتج اليورياز. يساعد انزيم اليورياز البكتيريا على استعمار معدة الإنسان من خلال استقلاب اليوريا من أجل تحييد بيئة المعدة. هدفت الدراسة الحالية إلى الكشف عن انتشار جين عامل الضراوة ureA للحلزونية البوابية، والتحقيق في تأثير هذا الجين على نتيجة اختبار اليورياز السريع (RUT). تم عزل ثمانين عينة خزعة من معدة المشاركين المشتبه في إصابتهم بالبكتيريا الحلزونية البوابية في مدينة أربيل. كان المشاركون 36 من الذكور و44 من الإناث واللذي تراوحت أعمارهم بين 18 و67 عامًا. أظهرت النتائج أن 42 (52.5٪) من المشاركين كانوا مصابين عند اختبارهم بواسطة RUT، بينما أظهر 59 (73.8٪) من المرضى عدوى بالبكتيريا الحلزونية البوابية عند اختبارهم عن طريق تفاعل البوليميراز المتسلسل (PCR) الذي استهدف جين S rRNA16. أظهرت نتائج اختبار تفاعل البوليميراز المتسلسل (PCR) المعتمد على الجين ureA أن 42 (52.5٪) من العينات كانت إيجابية. تتمثل النتيجة المهمة لهذا البحث في وجود توافق بنسبة 100٪ بين العينات الإيجابية فحص RUT و ureA. يمكن الاستنتاج من هذه الدراسة أن الشخص قد يكون مصابًا بالبكتيريا الحلزونية البوابية، لكن اختبار RUT يفشل في اكتشاف العدوى إذا كانت البكتيريا تفتقر إلى الجين ureA، مما يشير إلى وجود تأثير مباشر لهذا الجين على نتيجة RUT، وهو عيب في RUT.

INTRODUCTION

Helicobacter Pylori are spiral-shape flagellated bacteria with a length of 2 to 4 µm and a width of 0.5 to 1 µm [1, 2]. The prevalence of *H. pylori* varies greatly around the globe. Its highest prevalence occurred in Africa with about 70%, while the lowest one occurred in Switzerland (18%) [3]. *The pathogenicity of this bacterium* depends on different virulence factors, each of which performs a different biological function. In general, *H. pylori* virulence factors can be classified as belonging to one of three major pathogenic processes that include: colonization, immunological evasion, and disease induction [4].

The gastric condition acts as a limitation of the microbial growth. In order to colonize and overcome this gastric condition, *H. pylori* developed strategies like the production of urease enzyme that catalyzes the urea hydrolysis to form ammonia that neutralizes the gastric environment for the bacterial growth. Besides its important role in the colonization of the bacterium, it also acts as a potent immunogenic that elicits a vigorous immune response [5]. Infection with *H. pylori* can be diagnosed using different methods. Those diagnostic methods are classified into invasive methods and non-invasive methods. Each one has advantages and disadvantages. Molecular testing through PCR has high accuracy in detecting this bacterium and its virulence factor genes as well. PCR is considered accurate, simple, and rapid compared to other diagnostic methods. It can, in addition, be used for detecting different genes and their mutations [6]. Rapid urease test belongs to the invasive methods for detection of *H. pylori*. This test works through detecting of urease enzyme in the clinical specimens. Results of RUT are influenced by some factors like: urease concentration of the bacteria, temperature, time and condition of the optimal reaction, and substrate concentrations [7]. Generally, the main advantages of RUT include having high specificity of up to 99%, being rapid, simple, and cheap, and being the easiest test in a clinical setting. While there are several limitations and disadvantages to this test, like: low sensitivity of about 80%, that decreased more in patients with gastric bleeding, and the rate of false negative results increased in the case of antibiotics and achlorhydria. Besides those disadvantages, RUT may also give false positive findings through interfering with other bacteria that produce urease in the gastric mucosa [8, 9].

Furthermore, little attention has been given to the genotyping, virulence factors, and assessing the detection methods of *H. pylori* in Iraq, and particularly the Kurdistan region. Therefore, the aims of the study were to identify the prevalence of the *H. pylori's ureA* gene virulence factor and its relation with RUT, and also to detect this bacterium using molecular test PCR in comparison to RUT, which is the routinely used test among gastrointestinal patients in most hospitals in Erbil city.

MATERIALS AND METHODS

Samples collection

Eighty stomach biopsy samples were collected from participants who were admitted to the Hawler Gastroenterology and Hepatology Center at Rizgary Teaching Hospital in Erbil between November 2021 and January 2022. Of those, 36 were males (45%) and 44 were females (55%), aged 18 and above. The samples were collected through endoscopy of the upper gastrointestinal tract. The participants were asked to fast overnight and not to take any medication before the endoscopy. From each participant, two biopsy samples of about 0.5 cm were taken, one for the RUT and the other one was put in a 1.5 ml microcentrifuge tube that contained (1X) Dulbecco's phosphate buffered saline (DPBS) to be used for PCR. The samples were stored at -20 °C until DNA extraction [10].

DNA extraction

Genomic DNA was extracted from the tissue biopsy samples following the manufacturer's instructions of the DNA extraction kit (Presto™ Mini gDNA Bacteria Kit). Genomic materials were stored at -20°C until the PCR running. To evaluate the concentration and purity of the extracted DNA, a NanoDrop 1000 spectrophotometer was used in which 1µl of the genomic DNA was used and read at 260/280nm [11].

Polymerase chain reaction

The primers

Two sets of primers were used in this study, the first one targeted the *16S rRNA* gene, and was used to detect *H. pylori* in the biopsy samples [12]. The second primer set was used to detect the virulence factor gene *ureA* [13]. Both primers were checked by the National Center for Biotechnology Information (NCBI) software program (http://www.ncbi.nlm.nih.gov/BLAST). All of the primer sequences and the size of their products are listed in Table 1.

Table 1. The primers used in the present study.

	Target Gene name	Primer Name	Primer Sequence (5'-3') sequence	Product size (base pairs)	Annealing temp. (°C)	Reference
1	*16S rRNA*	16SR-F	ACTCCCTACGGGGGAAAGAT	141	59 ºC	[12]
2	*16S rRNA*	16SR-R	GGACCGTGTCTCAGTTCCAG			
3	*ureA*	ureA-F	TGATGGGACCAACTCGTAACCGT	244	60.2 ºC	[13]
4	*ureA*	ureA-R	CGCAATGTCTAAGCGTTTGCCGAA			

PCR running and conditions

A polymerase chain reaction using Gradient thermal cycler Alpha Cycler PCRmax series was carried out to amplify the selected genes (*16S rRNA* and *ureA*) using specific primers for each gene. The PCR mixture included 12.5µl of the master mix, 3 µl of the extracted DNA, 2.5µl of the forward primer, 2.5µl of the reverse primer, and the volume was completed to 25µl by adding nuclease free water [14].

The PCR running conditions for the first primer set (*16S rRNA*) and the second primer set (*ureA*) are shown in Table 2.

Table 2. The PCR cycling conditions for the primers used in the present study.

	Gene	Initial denaturation	45 cycles			Final extension
			Denaturation	Annealing	Extension	
1	*16S rRNA*	95°C/ 5 min	95°C/40 sec	59°C/45 sec	72°C/40 sec	72°C/5 min
2	*ureA*	95°C/3 min	95°C/45 sec	60°C/45 sec	72°C/2 min	72°C/ 5 min

Gel electrophoresis and result documentation

To observe the results of PCR amplification, agarose gel electrophoresis was carried out for the amplified samples. The 1.2% agarose gel was prepared by dissolving 1.2g of its powder in 100 ml of 1x TAE (Tris-acetate-EDTA) buffer, and 3µl of safe stain was added to the mixture, which was heated till boiling using microwave. Prior to pouring it into the gel chamber, a special comb was applied to make holes, and the gel was let to solidify at room temperature for approximately 30 min. Later, the comb was removed carefully and the chamber transferred to its place in the tank, which was filled with 1x TAE buffer. The first well of each gel was loaded with 5µl of a 100 bp DNA ladder, the second well for the negative control (a PCR reaction tube containing all the requirements except the samples' DNA), and the other wells for the samples, in which 6µl of the PCR products were loaded. Firstly, a power supply of 45 v/10 min was applied to avoid DNA shock, and then the voltage increased to 85 v/45 min. A UV-light illuminator at 240, 366 nm wave length was used to visualize the results of amplified DNA, and a 16MPX camera was used for documentation of gel pictures.

Statistical analysis

Data analysis was assessed using Fisher's exact test and the Turkeys Multiple Comparisons (One-Way ANOVA). For multivariate analysis, logistic regression was used. Variables with a p-value of less than 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSIONS

Results of the present study regarding gender showed that among 59 positive samples approved by PCR targeting *16S rRNA*, 26 (44%) were males and 33 (56%) were females. The 59 positive samples aged between 18 and 67, with a mean age of 39.65, of which 43 (72.9%) of them were below 50 years, and 16 (27.1%) of them were 50 years or older. Details of age and gender are shown in Table 3.

Table 3. Age and gender of the patients with positive *H. pylori* infection (n=59).

	Gender			Age		
	n=59	*n.*	*%*		*n.*	*%*
1	Male	26	44	< 50 years	43****	72.9
2	Female	33	56	> 50 years	16****	27.1
Total		59	100		59	100

***** (P<0.0001)*

Results of RUT among 80 samples demonstrated that only 42 (52.5%) of the suspected people had positive results, while 59 (73.75%) of the patients

showed positive results when tested by PCR targeting *16S rRNA* of *H. pylori*, as shown in Figure 1 and Table 4.

Table 4. Results of the RUT vs. the PCR targeting *16S rRNA* of *H. pylori* (n=80).

	Result	RUT		PCR (*16SrRNA*)	
	n=80	*n.*	*%*	*n.*	*%*
1	positive	42****	52.5	59****	73.75
2	negative	38	47.5	21	26.25
Total		80	100	80	100

***** (P<0.0001)*

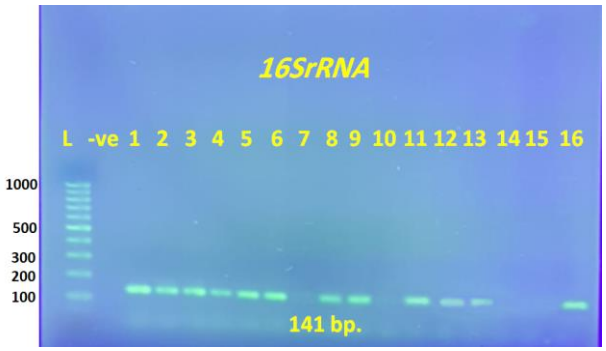

Figure 1. 1.2% Agarose gel electrophoresis analysis for the PCR products stained with safe stain shows partial amplification (roughly 141 bp) of the *16S rRNA* gene of *H. pylori*. Lane L: 100 bp DNA Marker; Lane -ve: negative control; Lanes 1-16: the PCR products, samples numbers 1 to 6, 8, 9, 11, 12, 13 and 16 are positive for *H. pylori*. The other samples: 7, 10, 14, and 15 are negative. The PCR was run on 85V for 45 min.

Regarding the detection of the *ureA* gene using PCR, among 59 positive *H. Pylori* cases, 42 (52.5%) of them were positive and had the *ureA* gene, as shown in Figure 2 and Table 5.

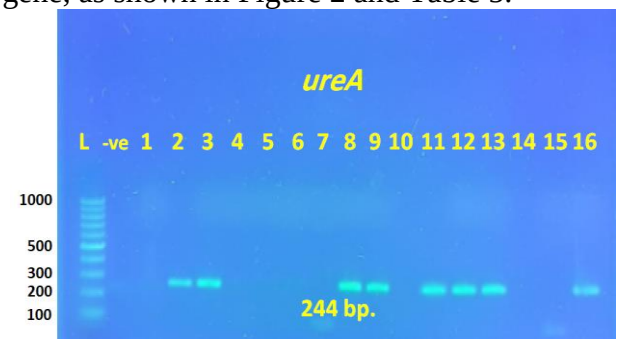

Figure 2. 1.2% Agarose gel electrophoresis analysis for the PCR products stained with safe stain shows partial amplification (roughly 244 bp) of the *ureA* gene of *H. pylori*. Lane L: 100 bp DNA Marker; Lane -ve: negative control; Lanes 1-16: the PCR products, samples numbers 2, 3, 8, 9, 11, 12, 13, and 16 are positive. The other samples: 1, 4 to 7, 10, 14, and 15 are negative for the *ureA* gene. The PCR was run on 85V for 45 min.

Table 5. Results of the PCR targeting *16S rRNA* vs. *ureA* for detection of *H. pylori* (n=80).

	Result	PCR (*16S rRNA*)		PCR (*ureA*)	
	n=80	*n.*	*%*	*n.*	*%*
1	positive	59****	73.75	42****	52.5
2	negative	21	26.25	38	47.5
Total		80	100	80	100

***** (P<0.0001)*

According to the statistical analysis, there were non-significant differences regarding gender with a P-value of P<0.1863, but there were highly significant differences regarding age groups (P<0.0001).

Moreover, highly significant differences (P<0.0001) were reported between the results of the RUT and the PCR for the diagnosis of *H. pylori*. Regarding the *ureA* gene, it significantly differed from *16S rRNA* (P<0.0001) but the results of the first gene were not significantly different (P<0.9999) from those of RUT. In each sample where the *ureA* gene was positive using PCR, RUT was positive too, and wherever *ureA* was negative in PCR, RUT was also negative, indicating 100% compatibility between RUT and PCR based on the *ureA* gene (Pearson r 1.00) as shown in Figure 3 and Table 6.

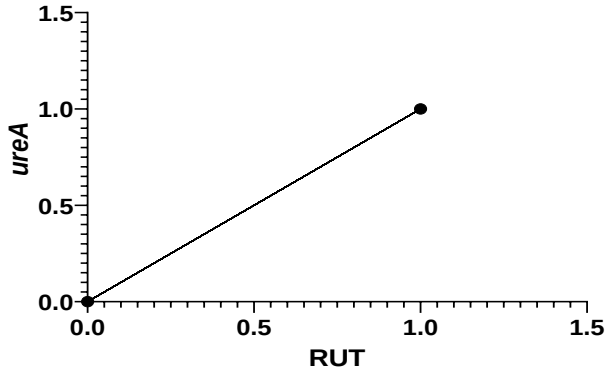

Figure 3. The correlation (Pearson r) between RUT with PCR based on *ureA* among *H. pylori* isolates.

Table 6. The correlation (Pearson r) between RUT with PCR based on *ureA* among *H. pylori* isolates.

Correlation analysis	RUT vs. *ureA*
Pearson r	1.00
P value	>0.9999
P value summary	ns
Significant (p-value < 0.05)	No

For discussions, regarding gender, numbers of females were slightly higher than males, results of statistical analysis showed no significant differences. According to a recent study carried out by Miranda and his colleagues in 2019, they detected a higher prevalence among females than males, which agreed with the results of the present study [15]. Another meta-analysis study that included results from several research projects from 2013 to 2017, also showed the prevalence of *H. pylori* among females rather than males [16].

Results of *H. pylori* regarding ages showed high significant differences between the two groups of this study, in which 43 (72.9%) of the patients were below 50 years of age and 16 (27.1%) of them were above 50 years of age. Nevoa *et al.*, (2017) found that 68.24% of their patients were below 50 years of age and 31.76% were above 50 years of age, their findings were somehow close to the present study [11]. Also, Hong *et al*., (2019) in their meta-analysis study, detected a high prevalence of *H. pylori* in the mid-thirties individuals, this agrees with the present study findings [16].

Regarding results of RUT and PCR, among 80 samples, only 42 (52.5%) showed positive results for RUT, while 59 (73.75%) were positive for PCR targeting *16S rRNA*. Statistical analysis showed highly significant differences ($P<0.0001$) between these two tests for the detection of *H. pylori*. Such a result was also confirmed by many other studies that found that PCR has a higher sensitivity and specificity than RUT [11, 13]. *PCR* is more sensitive and accurate than RUT, as the last one could be affected by several factors that reduce its accuracy. Size and location of the biopsy, bleeding, taking medications and the presence of other bacteria that have urease activity that can give a false positive result for RUT are among those factors [8, 17, 18].

Regarding the *ureA* gene, out of 80 cases, only 42 (52.5%) of the samples were positive for the *ureA* gene that encodes for an important virulence factor, which is urease enzyme, this ensures that *H. pylori* has the ability to resist the acid environment of the stomach [11]. But it should be considered that not all *H. pylori* strains possess the *ureA* gene [19]. According to the research of Deenonpoe and his colleagues in 2017, they detected *ureA* with a frequency of 64.6% in *H. pylori* strains, their result agrees with the present study [20]. While another research conducted by Alnaji and Omran (2018) in Babylon Province, Iraq, the *ureA* gene was detected in this bacterium with a high frequency of 89.8% [21]. In addition, Nevoa *et al.*, (2017) in Brazil detected *ureA* in 82.35% of the positive *H. pylori* samples [11]. While low frequencies (39.28%) of *ureA* were reported by Jabbar *et al*., (2015) in AL Samawa and AL Diwaniya provinces, Iraq [22]. Wahab *et al*., (2015) carried out a research in Pakistan, and detected *ureA* in 50% of the *H. pylori* strains [23].

Differences in the frequencies of the *ureA* gene between the present study and other studies could be due to different reasons, including geographical distribution. A study carried out by Correa and Piazuelo, in 2012, revealed that *H. pylori* strains showed genetic diversity around the world. This is due to migrations and, subsequently, geographic and ethnic distribution between human groups [24]. Furthermore, sample size and period of sampling are among the factors that have an impact on the distribution of different *H. pylori* strains and the frequencies of the virulence factors among these different strains [25, 26].

The important finding of this research showed that wherever the *ureA* gene was detected as positive, RUT also showed a positive result, and the contrast was also true. Results of statistical analysis showed a 100% correlation between RUT and PCR targeting *ureA*, indicating the direct relationship and effect of the *ureA* gene on the result of RUT. According to the present study, those strains of *H. pylori* that lack the *ureA* gene cannot be detected through RUT, while they can be detected by PCR targeting *16S rRNA*. This point could be added as a new limitation for RUT, in which the result of this test depends on the strain of *H. pylori* that produces urease enzyme. This finding needs further investigation as it makes RUT suspicious for detecting different strains of *H. pylori*. Likewise, this could be part of the answer to the question of why PCR detects higher rates of *H. pylori* when RUT fails.

CONCLUSIONS

Based on the obtained results, it can be concluded that PCR is more sensitive and accurate than RUT for detecting *H. pylori*. RUT, by having several limitations, could not be relied upon and trusted for detecting *H. pylori*. The results of RUT depend on the presence and expression of virulence factor genes in the *H. pylori* strain. Despite its importance, the *ureA* gene shows variability among different *H. pylori* strains. The *ureA* gene has a direct impact on the result of RUT.

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