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Original article

Detection of *hefA* gene in multidrug resistant *Helicobacter pylori* at Tanta University Hospital

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ABSTRACT

Background: The evident multidrug resistance (MDR) associated with *Helicobacter pylori (H. pylori)* is a serious public health problem. Multidrug resistance was defined as resistance to at least one antimicrobial agent in three or even more antimicrobial classes. The *hefA* gene encodes an active efflux process against antimicrobials, and its expression in *H. pylori* may contribute to the upsurge of resistant strains. **Aim of the study:** Detection of MDR *H. pylori* among endoscopic isolates and examining the role of *hefA* gene expression in the occurrence of MDR *H. pylori*. **Methods:** Our research involved 40 *H. pylori* endoscopic isolates using conventional microbiological methods. We identified MDR in *H. pylori* isolates using E testing procedures for metronidazole, ciprofloxacin, amoxicillin as well as clarithromycin. We detected the *hefA* gene expression among the isolated *H. pylori* by real-time PCR. **Results:** out of 40 isolates of *H. pylori*, 13 (32.5%) were MDR and 27 (67.5%) were not MDR. We found *hefA* gene in MDR *H. pylori* isolates with total (mean ± SD) equal to 7.055385±2.591111, but in *non*-MDR *H. pylori* isolates were with total (mean ± SD) equal to 2.591111±0.720189. There was a statistically significant difference in *hefA* expression levels among both MDR *H. pylori* isolated strains and non-MDR ones; the *p* value was 0.001. **Conclusion:** The *hefA* gene expression in *H. pylori* plays an important role in the emergence of MDR *H. pylori* strains as one of the genes encoding for efflux pump mechanisms.

Introduction

Helicobacter pylori (H. pylori) is a microaerophilic Gram-negative spiral (helical) bacterium. It is named *Helicobacter* due to its helical shape [1]. Its helical shape aids in pathogenicity by allowing it to invade the gastric mucosa as well as establish infection [2]. In 1982, Australian doctors Barry Marshall and Robin Warren were the first to recognize *H. pylori* [3]. *Helicobater pylori* is a major pathogen since it can cause not only stomach cancer but also oesophageal as well as colorectal cancer [4]. A high percentage of patients with *H. pylori* infection (about 90%) usually do not have any symptoms or

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complications [5]. However, about 10% of these patients may have the risk of developing peptic ulcers and some cases may proceed to develop cancer [6].

Helicobacter pylori can survive in the stomach's high acidity due to a variety of factors, including the presence of its flagella, which allows it to penetrate deep into the gastric mucosal lining and reach the underneath epithelium, which is less acidic [7]. *Helicobacter pylori* detects the pH gradient in the mucus and keeps moving towards the less acidic region via cytokine production; it also produces adhesins, that further bind to lipids and carbohydrates in the epithelial cellular membranes; and it neutralizes its environmental acidity by generating huge amounts of urease, which transform the gastric urea into carbon dioxide and ammonia [8].

There are numerous methods for detecting *H. pylori*, such as endoscopic biopsy followed by histopathological examination and/or microbiological examination. As the endoscope is an invasive method that is not recommended as the first diagnostic maneuver, serum antibody, antigen detection, and the carbon urea breath test can all be used as non-invasive diagnostic techniques for *H. pylori* [9]. A histological examination from two sites after endoscopic biopsy, combined with either a rapid urease test or microbiological culture, has been documented as the most precise method for the *H. pylori* detection [10].

Helicobacter pylori was sensitive to most common antibiotics, and it was easy to eradicate, especially if the patient took the accurate treatment for an adequate dose and duration. The development of antibiotic resistance is by far the most common cause of therapeutic failure in *H. pylori* infections [11]. Reduced drug uptake or increased drug efflux is a frequent cause of inherent drug resistance in *H. pylori* [12]. The efflux concept is commonly observed in *H. pylori* [13]. There are five different families of active efflux transporters identified in bacteria; the most common of these is the resistance nodulation cell division (RND) family [14]. This family consists of three members: inner membrane efflux proteins (IEPs), periplasmic efflux proteins (PEPs), and outer membrane efflux proteins (OMEPs) [15].

Three superfamilies of RND efflux systems, chiefly *hef*ABC, *hefDEF*, and *hefGHI*, were discovered. They were indeed made up of a translocase accessory protein as well as a TolC isoform. TolC homologs encoding the outer membrane efflux protein are *hefA*, *hefD*, and *hefG* [16].

Several therapeutic approaches are available for *H. pylori*, which can be treated with the combined effect of proton pump inhibitors (PPIs) and various antibiotics. Proton pump inhibitors is coupled with amoxicillin and clarithromycin in first-line triple therapy. In areas where rate of clarithromycin resistance is greater than 15%, triple therapy with metronidazole and otherwise bismuth-containing quadruple therapy is highly suggested [17].

Triple, quadruple, quintuple or rather sextuple drug resistance were discovered. Multi drug resistance prevalence has ranged both between and within countries. Not only national antibiotic usage, misapplication, therapeutic failures, but also bacterial factors such as mutations, active efflux, and biofilms have all been interconnected to MDR [18].

The purpose of this study was to detect the presence of MDR *H. pylori* among the isolated *H. pylori* from endoscopic samples and examine the role of *hefA* gene expression in the occurrence of MDR *H. pylori* as one of genes encoding the efflux pump mechanism in *H. pylori.*

Patients and Methods

Study design and patients

This was a cross-sectional study of 50 patients admitted to Tanta University Hospitals' Tropical Medicine and Infectious Diseases Department during the period of the study, which was from Feb. to August 2022. Patients who had severe or frequent stomach aches or digestive symptoms like indigestion, nausea, and/or vomiting in the previous three months, those who were prescribed an endoscopic investigation, in addition to those who hadn't even received any prior prescribed medication for *H. pylori*, were all eligible for our study. On the other-hand, patients with a recent history of *H. pylori* medications in less than 3 months before the endoscope, patients with a history of previous allergic reactions to antibiotics or any contraindication to medicinal drugs, and patients with intestinal bleeding were excluded.

Sampling

Biopsy specimens were obtained from the antral and oxyntic gastric mucosa during endoscopy and quickly transported to the microbiology laboratory.

Conventional microbiological diagnosis

The biopsy specimens for culture were quickly transported to the microbiology laboratory and processed within a few hours [the samples were transported in a transport medium (Stuart's media)]. We did rapid urease test [test was performed at the time of gastroscopy]. A biopsy of mucosa was taken from the antrum of the stomach, and was placed into a medium containing urea and an indicator such as phenol red. The urease produced by *H. pylori* hydrolyzes urea to ammonia, which raises the pH of the medium, and changes the color of the specimen from yellow (negative) to red (positive)],The biopsy specimens were homogenized by grinding them between two sterile microscopic slides and mixing them with sterile saline before being cultured on Columbia agar plus 7% defibrinated blood and DENT supplement (vancomycin, trimethoprim, amphotericin B, and cefclidine) [Oxoid,UK]and incubated for 3-4 days under microaerophilic conditions (5 % O2, 10 % CO2). This microaerophilic condition was caused by the 2.5 L microaerophilic gas-producing pack [No: HBYY008]. Product description: 2.5L microaerophilic gas producing bag was used for construct micro-aerobic environment to culture microaerophilic microbes. Procedure: we took an anaerobic culture bag or box, we inserted 4-10 plates inoculated microaerophilic bacteria, we took a 2.5L microaerophilic gas producing pack, we cut the aluminum foil, we removed the microaerophilic gas producing bag quickly, and then we sealed anaerobic culture bag. Based on colony morphology, gram staining, and biochemical reactions such as catalase and oxidase, and urease tests, clinical isolates were identified as *H. pylori*. For further PCR, pure *H. pylori* isolates were stored at -80 °C in brain-heart infusion broth with glycerol [19].

E test for antimicrobial susceptibility to isolated *H. pylori* strains (Himedia labs.,India)

An E test on Mueller-Hinton agar reinforced with 10% blood had been used to determine the minimal inhibitory concentrations (MICs) of isolates for metronidazole, clarithromycin, ciprofloxacin, and amoxicillin. Following incubation, MIC was detected using EUCAST breakpoints defined resistance as follows: MIC >0.12 mg/L for amoxicillin, >8 mg/L for metronidazole, >0.5 mg/L for clarithromycin, and >1 mg/L for ciprofloxacin [20]. We used E test for detection of multidrug resistance among isolated *H. pylori* as following: MDR was defined as resistance to at least one antimicrobial agent in three or even more antimicrobial classes [21].

HefA* gene expression detection employing real-time PCR in isolated *H. pylori

Total RNA was isolated from tissue isolate as per the manufacturer's instructions using the Gene JET RNA Purification Kit (Thermo Scientific, #K0731, USA). The obtained RNA had been kept at - 80 °C. RevertAid H Minus Reverse Transcriptase (Thermo Scientific, #EP0451, USA) had been used to generate cDNA as per the manufacturer's protocol. Until the expression laboratory activity, the cDNA was stored at -80 °C. The *hefA* gene had been studied in separated *H. pylori* strains in comparison to gyrB (a housekeeping gene encoding for gyrase B). The Step One real-time PCR system was used for real-time quantitative PCR (Applied Biosystems, CA). To prepare the final volume of 25 µl PCR mix, 2 µl of cDNA product was mixed with 12.5 µl of 2 Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, #K0221, MA), 1 µl forward primer, 1 µl reverse primer, and 8.5 µl of nuclease-free water. The sequence alignments of the genes from *H. pylori* 11637 in GenBank were used to design the gene-specific primers. The sequences of *hefA* (accession no. AF059041) are F: (5'-ACGCCTCGAGTAAAAGCG CAAGGGAATTTG-3') and R: (5'-ACGCTCTAG ATTCGCTAATTGGCCTAGCAT-3'). The PCR primers were predicted to amplify a 162-bp amplicon. The sequence of *gyrB* (accession no. AB084049) is F: (5'-TTACTACGACTTATCCTGGGGCTA GCGCTG-3') and R: (5'-CCCATCAATTTCCACAT TCTCCGC-3'). A 267-bp amplicon was predicted to be amplified by the PCR primers. The amplification reactions were first denatured at 95 °C for 3 minutes, followed by 40 cycles of DNA denaturation at 95 °C for 10 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 45 seconds. Finally, for melting curve analysis, the temperature was raised from 63 to 95 °C. The cycle threshold (CT) values for the target and reference genes were calculated. The relative concentration of *hefA* gene expression in each sample was calculated using the comparative method formula $2^{-\Delta CT}$.

Results

Our study included 50 patients who underwent GIT endoscopy at Tanta University's Faculty of Medicine's Tropical Medicine and Infectious Diseases. Of these 50 endoscopic samples, 40 (80 %) *H. pylori* isolates were isolated using conventional microbiological methods; 13 (32.5 %) were MDR isolates, and 27 (67.5 %) were not MDR as shown in **figure (1),** according to E test results used for detection of MDR among isolated *H. pylori*. E test results are shown in **table (1);** 15 (37.5%) isolates were sensitive to all tested antibiotics, 2 (5%) isolates were resistant to metronidazole only, 2 (5%) isolates were resistant to amoxicillin only, 8 (20%) isolates were resistant to clarithromycin only, 10 (25%) isolates were resistant to metronidazole, clarithromycin, and amoxicillin, 3 (7.5%) isolates were resistant to both clarithromycin and ciprofloxacin. In our study, we used RT real-time PCR to look for *hefA* gene expression in isolated *H. pylori*. We found it in MDR *H. pylori* isolates with total (mean ± SD) equal to 7.055385±2.591111, but in *non*-MDR *H. pylori* isolates were with total (mean ± SD) equal to 2.591111±0.720189, so *hefA* gene was more expressed in MDR *H. pylori* than in non MDR *H. pylori*, and the distinction in *hefA* gene expression between MDR *H. pylori* isolates and *non*-MDR *H. pylori* isolates has been statistically significant, with a *p* value of 0.001*, as shown in **table (2).**

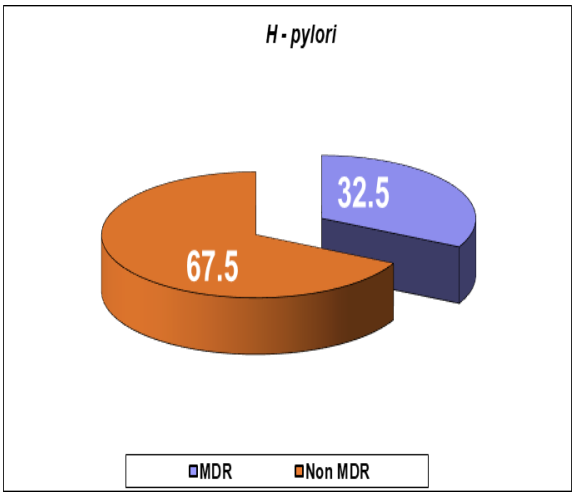
Table 1. Results of E test done for 40 *H. pylori* isolates.

Metronidazole	Clarithromycin	Ciprofloxacin	Amoxicillin
S	S	S	S
S	S	S	S
S	S	S	S
S	S	S	S
S	S	S	S
S	S	S	S
S	S	S	S
S	S	S	S
S	S	S	S
S	S	S	S
S	S	S	S
S	S	S	S
S	S	S	S
S	S	S	S
S	S	S	S
R	S	S	S
R	S	S	S
S	R	S	S
S	R	S	S
S	R	S	S
S	R	S	S
S	R	S	S
S	R	S	S
S	R	S	S
S	R	S	S
S	S	S	R
S	S	S	R
R	R	S	R
R	R	S	R
R	R	S	R
R	R	S	R
R	R	S	R
R	R	S	R
R	R	S	R
R	R	S	R
R	R	S	R
R	R	S	R
S	R	R	S
S	R	R	S
S	R	R	S

Table 2. The findings of the *hefA* gene in *H. pylori* isolates.

***HefA* gene**	**MDR *H. pylori* (13)**	**Non MDR *H. pylori* (27)**
	8.52	2.32
	10.32	1.74
	8.32	3.21
	4.51	2.52
	7.45	3.52
	5.63	2.74
	6.52	2.65
	3.62	3.85
	3.74	3.45
	10.85	1.42
	7.45	3.69
	6.67	2.52
	8.12	3.45
		2.85
		1.45
		3.12
		2.25
		2.95
		3.65
		2.15
		1.85
		1.98
		2.47
		2.34
		1.52
		2.05
		2.25
Mean	7.055385	2.591111
SD	2.272765	0.720189
T test	8.072	
***P* value**	0.001*	

Figure 1. The percentage of the isolated MDR *H. pylori* in our study.



Discussion

Multi drug resistant *H. pylori* was found among isolated *H. pylori* in our study. **Asif et al.** [21] and **Ge et al.** [22] discovered the emergence of MDR *H. pylori* in their studies. And this emergence represented a major health problem, as it affected greatly the treatment options used for treatment of GIT symptoms due to infection with *H. pylori*. *Helicobacter pylori* infection treatment usually entails taking two or more antimicrobials at the same time. However, due to the emergence of antimicrobial resistance, it may eventually be insufficient. The efflux pump, which exports antimicrobials out of *H. pylori*, is one of the most important mechanisms of antimicrobial resistance in *H. pylori*, and this efflux pump usually results in multidrug resistance [23, 24].

Efflux pump composed of large transport proteins, which present on the bacterial cell membrane, and this efflux pump played an important role in the extrusion of xenobiotics (Comprising neurotransmitters, toxic substances, dyes, antibiotics). Based on the amino acid sequence and the source of energy utilized (ATP or hydrogen / sodium ions). The efflux pump is studied widely in many bacteria but this efflux pump needs more studies in *H. pylori*. So in our study we studied the role of *hefA* gene expression in the emergence of MDR *H. pylori* as one of the most important genes encoding for efflux pump in *H. pylori* [22].

RND is an active transporter superfamily with 1000 amino acids [25]. Multi drug resistance is caused by RND efflux in *H. pylori*. Whereas most efflux systems exist in the cytoplasm and extrude drugs to the periplasm, RND is the only efflux system that traverses the inner and outer membranes and is powered by the three component systems [26], making it superior to other smaller pumps by extruding drugs directly to the exoplasm [27, 28]. *Helicobacter pylori* was found to have three operons encoding RND efflux systems: hp0605-hp0607as *hefA*BC, hp0969-hp0971as hef FED now known as czn ABC, and hp1329-h1327as, czc A, czc B, crd B.

The *hefA* gene was chosen for this study from three TolC homologs: *hefA*, *hefD*, and *hefG*, which encode the outer membrane efflux protein in *H. pylori* because it is the most commonly expressed gene in *H. pylori* strains among these homologs, while the *hefD* and *hefG* genes are weakly expressed in vitro, and *hefA* gene was considered as the only RND efflux systems known to have a broad range of antibiotic substrates [23, 29].

We examined the isolated *H. pylori* for the presence of the *hefA* gene by real time PCR because it is one of the most important genes encoding the efflux pump mechanism in *H. pylori* [30]. We used RT real-time PCR to look for *hefA* gene expression in isolated *H. pylori*. We found it in MDR *H. pylori* isolates with total (mean ± SD) equal to 7.055385±2.591111, but in *non*-MDR *H. pylori* isolates were with total (mean ± SD) equal to 2.591111±0.720189, so *hefA* gene was more expressed in MDR *H. pylori* than in non MDR *H. pylori* ,the distinction in *hefA* gene expression between MDR *H. pylori* isolates and non-MDR *H. pylori* isolates has been statistically significant, with a *p* value of 0.001*, and this was in agreement with **Ge X et al.** as well as **Zhi-Qiang L. et al.** who also found a significant increase in *hefA* gene expression among the isolated MDR *H. pylori* strains and non MDR ones [22, 31].

The emergence of MDR *H. pylori* affects seriously the available protocols for *H. pylori* treatment as there are six common antibiotics (clarithromycin, levofloxacin, metronidazole, tetracycline, amoxicillin, and rifabutin) are used for treatment of *H. pylori* infections. Together with combination of a PPI, later-arise of MDR strain necessitates alternation in treatment. The second line treatment included fluoroquinolone combined with PPI. The reported resistance to quinolones like ciprofloxacin, levofloxacin, and moxifloxacin have raised up to 38.2%, 37.7%, and 34.6% in the Korean population, compared to previous reports. When there is a fluoroquinolone resistance, this is replaced with rifabutin containing therapy or even a combination of bismuth with various antibiotics. Bismuth third-line therapy includes a combination of two drugs with PPI and a bismuth-based quadruple approach [22].

So, as we detected a significant high expression of *hefA* gene among the isolated MDR *H. pylori* than in non MDR *H. pylori* isolates , and *hefA* gene is a one of genes which encoding efflux pump of antibiotics in *H. pylori* , thus our results indicated that efflux pump mechanisms play an important role in emergence of multidrug resistance in *H. pylori*.

Conclusion

We detected the emergence of MDR *H. pylori* among the isolated *H. pylori* from endoscopic samples, and the *hefA* gene expression plays a critical role in the occurrence of *H. pylori* MDR as we detected a statistically significant increase in its expression among isolated MDR *H. pylori* and thus indicated the role of efflux pump mechanisms in this multidrug resistance emergence.

Limitations in our study

The great effort was done with the isolation of *H. pylori* by conventional microbiological methods, because *H. pylori* is one of fastidious bacteria so we used transport media for transporting the endoscopic samples, and we processed the samples within few hours in the microbiological lab, also it needs special conditions during culturing, which costed us time and money, we did also rapid urease test, which must be at the time of the endoscopic sampling, so we attendeded the endoscopic sampling. We used endoscopic sampling which is an invasive technique to isolate *H. pylori* by conventional microbiological methods, which also represented a difficulty in our study.

Sample size calculation

The sample size and power analysis was calculated using Epi-Info software statistical package created by World Health organization and center for Disease Control and Prevention, Atlanta, Georgia, USA version 2002. The criteria used for sample size calculation were as follows: 95% confidence limit, 84% power of the study, the sample size was found at N = 50 sites.

Compliance with ethics requirements

All procedures were carried out in accordance with the responsible committee on human experimentation's ethical standards (institutional and national).

Conflict of interests

The authors state that they have no competing interests.

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