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Original article

Detection of antimicrobial resistance genes of *Helicobacter pylori* strains to clarithromycin, metronidazole, amoxicillin and tetracycline among Egyptian patients

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ABSTRACT

Background: Antibiotic resistance of *Helicobacter pylori* (*H. pylori*) treatment is on the rise, and is affecting the efficacy of current used therapeutic regimens.
Aim: We aimed to enhance the understanding of antimicrobial resistance rates of *H. pylori* strains recovered from patients at Theodor Bilharz Research Institute Hospital in Egypt, as a mandatory step before starting treatment.
Subjects and methods: Mutant genes conferring metronidazole, amoxicillin, clarithromycin, and tetracycline resistance were detected in 60 *H. pylori* strains recovered from patients who underwent upper endoscopic examination. Patients were considered to be infected with *H. pylori* when rapid urease test and detection of *16S rRNA* in gastric biopsies recorded positive. Molecular detection of resistant genes to metronidazole (*rdx* gene) and amoxicillin (*pbp1A* gene) was carried out by conventional PCR followed by sequencing of PCR products. While detection of *23S rRNA* gene conferring clarithromycin resistance and detection of *16S rRNA* mutation gene conferring tetracycline resistance were carried out by real-time PCR.
Results: H. pylori resistance rates to metronidazole, and amoxicillin were 25% and 18.3% respectively. While for clarithromycin and tetracycline, point mutations in *23S rRNA* types *A2142G* and *A2143G* and in *16S rRNA* of *H. pylori* were assessed by real time PCR assay respectively. Resistance mutant genes were found to be 6.7% of clarithromycin and 1.7% of tetracycline. Combined resistance rates to metronidazole and amoxicillin was (11.6%) followed by metronidazole and clarithromycin (5%), while patterns of clarithromycin and amoxicillin (1.6%), metronidazole, clarithromycin and amoxicillin (1.6%) were revealed.
Conclusion: Data concerning antimicrobial resistance genes play an important role in empiric treatment of *H. pylori* infection. According to our results, *H. pylori* resistance to metronidazole and amoxicillin was relatively high. Clarithromycin is still a good option for first line anti-*H. pylori* treatment. Combined resistant strains are emerging and may have an effect on the combination therapy.

1. Introduction

Eradication of *H. pylori* does not only heal gastritis of peptic ulcer disease, but may prevent the spread of infection and *H. pylori* recurrence. Also, it may reduce the risk of development of gastric cancer, thus reducing further costs required for the treatment of subsequent *H. pylori*-associated diseases [1]. The treatment of *H. pylori* infection employs a triple drug regimen using one of the following antibiotics (amoxicillin, tetracycline or clarithromycin)

Abbreviations: H.pylori, *Helicobactor pylori*; PPI, Proton pump inhibitor; TBRI, Theodor Bilharz Research Institute; DNA, Deoxy-ribonucleic acid; PCR, Polymerase chain reaction; *16S rRNA*, 16 S ribosomal ribonucleic acid; dNTPs, Deoxynucleotide Triphosphates; SPSS, Statistical Package for Social Sciences; Bp, Base pair; Lab, Laboratory; Ul, Micro-litre; min, Minute; UV light, Ultraviolet light; Pmol, Pico-mole; Tm, Temperature.

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along with metronidazole and a proton pump inhibitor (PPI) or bismuth salt, and combined PPI and bismuth salt with quadruple regimen when triple therapy regimens have failed. In Egypt, the standard therapy combines PPI and metronidazole, and one antimicrobial drug, chosen from among clarithromycin and amoxicillin [2–4].

Antibiotic resistance is a key factor in the failure of eradication therapy and recurrence of *H. pylori* infection [5]. Worldwide *H. pylori* antibiotic resistance towards different antibiotics is increasing and it is the main factor affecting efficacy of current therapeutic regimens. Prevalence of bacterial resistance varies in different geographic areas, and it has been correlated with the widespread use of certain antibiotics in the general population (i.e. clarithromycin for respiratory infections, metronidazole for parasite diseases and dental infections, tetracycline for respiratory and bowel diseases, amoxicillin for streptococcal pharyngitis, urinary tract infections [6]. In countries where the use of clarithromycin is rare, the resistance rate is low, while in countries where its use is wide, the resistance rate reaches 10–15%. Similarly, metronidazole-resistance rate is much higher in developing countries (50–80%) [7]. Amoxicillin is suggested for anti-*H. pylori* triple therapy in regions where metronidazole resistance is high. Universal resistance to amoxicillin is uncommon; it was detected in 14.67% [8].Tetracycline resistance has not become a great problem yet [2].

The aim of this study was to enhance the understanding of antimicrobial resistance rates of *H. pylori* strains recovered from patients at Theodor Bilharz Research Institute TBRI Hospital in Egypt, as a mandatory step before starting treatment.

2. Subjects and methods

2.1. Patients and clinical specimens

From December 2015 to January 2017, 60 *H. pylori* infected patients who underwent upper endoscopy for various dyspeptic symptoms at Endoscopy Unit, TBRI Hospital were enrolled in this study. The sample size was calculated by Epi Info program (version 6.0) at 95%Confidence Limit, Power of the Test is 80% and Alpha Setat 0.05 (Type Error). None of the patients had received non-steroidal anti-inflammatory drugs, as well as antibiotics, H_2 receptors antagonists or proton pump inhibitors in the past four weeks prior to the study. During upper gastrointestinal endoscopy, four antral biopsies were obtained from each patient. One biopsy was tested for rapid urease test, that was performed using rapid urease liquid test kit (Bussero, Milan, Italy) and the other three gastric biopsies were stored in sterile physiological saline and kept at −70 °C until processed. DNA extraction used directly for detection of *16S rRNA*, *rdxA, Pbp1*, *23S rRNA* and *16S rRNA* mutation genes using PCR assays. A patient was considered to be infected with *H. pylori* when he had positive rapid urease test and confirmed by detection of *16S rRNA* in gastric biopsy specimens. This work was supported by Theodor Bilharz Research Institute (TBRI) as a part of an ongoing internal project No. 93 T. The protocol was approved by TBRI institutional review board (FWA00010609) and all patients provided a written informed consent. The work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for Experiments in Humans.

2.2. DNA extraction from gastric biopsy specimens and PCR assays

Genomic DNA was extracted from gastric biopsy specimens using QIAamp tissue kit (QIAamp DNA Mini Kit from QIAGEN, USA), following manufacturer guidelines.

2.2.1. Detection of H. Pylori 16S rRNA gene

Conventional PCR assay was performed in a volume of 50ul with approximately 5 μg of extracted DNA, 200 μM (each) dNTPs, 25 pmol for each primer, 1.5 μM Magnesium Chloride and 1unit (U) of *Taq* polymerase (Gotaq Flexi DNA, M8305, Promega, Inc., USA) in PCR buffer using primers according to[9]. The sequences of forward (F) and reverse (R) primers are: F 5′-CTG GAG AGA CTA AGC CCT CC-3′ and R 5′-ATT ACT GAC GCT GAT TGT GC-3′.

An initial denaturation at 95 °C for 5 min followed by 35cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min and 72 °C for 1 min. This was followed by a final extension at 72 °C for 5 min. The size of the expected amplicon was 110 bp.

2.2.2. Detection of rdxA gene conferring metronidazole resistance

Conventional polymerase chain reaction (PCR) assay was performed in a volume of 50ul with approximately 2.5 μg of the extracted DNA, 200 μM (each) dNTPs, 2.5 μM Magnesium Chloride and 0.25 U of *Taq* polymerase (Promega®, USA) in PCR buffer using 25 pmol primers. Primer sequences according to (Debets-Ossenkopp et al. [10] are: F: 5′-AAT TTG AGC ATG GGG CAG A-3′ and R: 5′-GAA ACG CTT GAA AAC ACC CCT-3′.

Amplification was carried using; denaturing temperature at 95 °C for 5 min, followed by 40 cycles denaturing temperature at 94 °C for 30 s, an annealing temperature at 55 °C for 30 s and an elongation temperature at 72 °C for 1 min. This was followed by a final extension of 72 °C for 10 min. The size of expected amplicon was 581 bp.

2.2.3. Detection of Pbp1A gene conferring amoxicillin resistance

Conventional PCR assay was performed in a volume 50ul, with approximately 1 μg of extracted DNA, 200 μM (each) dNTPs, (Table: 3), 0.5u of *Pfu* DNA polymerase (Thermo®, USA) in PCR 5 μl *Pfu* buffer with Magnesium Sulphate and 25 pmol primer [12]. The sequences of the F and R primers are: F 5-GCG ACA ATA AGA GTG GCA-3′ and R 5′-TGC GAA CAC CCT TTT AAA T-3′.

The following cycling parameters were used; denaturing temperature of 95 °C for 3 min, followed by 35 cycles of denaturing temperature of 95 °C for 1 min, an annealing temperature of 54 °C for 1 min and an elongation temperature of 72 °C for 5 min and final extension at72°C for 10 min. The size of expected amplicon was 2300 bp.

Each PCR product was separated on 2% agarose gel with ethidium bromide, and 100 bp ladder used as DNA molecular weight standard. In each PCR assay, a negative control (lacking DNA) was included. PCR products were analyzed under UV light [11].

2.2.4. Sequencing of DNA fragments of rdxA and Pbp1A genes

Genomic DNA fragments were purified from agarose gel using purification kit (Thermo Scientific GeneJET Gel Extraction Kit, USA) according to the manufacturer's instructions. DNA sequencing was made by Sanger sequencing method following the manufacturer's instructions. Based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication [12].

To compare between wild and mutant genes, alignment was made online at site http://www.ebi.ac.uk/Tools/msa/clustalw2/.

2.2.5. Detection of 23S rRNA gene conferring clarithromycin resistance

The real-time (TaqMan, USA) PCR assay was performed in a volume of 25ul, with approximately 10 ng of purified DNA, 12.5 μl universal PCR master mix (ABI, USA), 36 ul of each primer, 8 μM of FAM- and VIC-labeled TaqMan MGB probes [13].

Primers and probes sequences according to Lins et al. [14] are:
F: 5′ TCA GTG AAA TTG TAG TGG AGG TGA AAA-3′
R: 5′ CAG TGC TAA GTT GTA GTA AAG GTC CA-3′
VIC

AAG ACG GAA AGA CC (for DNA wild type)
FAM
AAG ACG GGA AGA CC (for mutation: 23S rRNA A2142G)
FAM
CAA GAC GGA GAG ACC (for mutation: 23S rRNA A2143G)

Two point mutations of *H. pylori 23S rRNA* (*A2142G* and *A2143G*) were assayed. The conditions of Real-time PCR amplification were 95 °C for 10 min and 40 cycles of 92 °C for 15 s and 60 °C for 1 min. The result of the analysis yields two major clusters corresponding to the three genotypic constituents: (i) wild-type homozygous (ii) mutated type homozygous and (iii) heterozygous.

2.2.6. Detection of 16S rRNA mutation gene conferring tetracycline resistance

The real-time (TaqMan) PCR assay was performed in a volume of 25ul, with approximately 1 ug of extracted DNA, 12.5 ul universal PCR master mix (Applied Bio-systems ABI, USA), 5 pmol of each primer and 2.5 pmol of probe [15].

The sequence of the F and R primers are: F 5′-CGG TCG CAA GAT TAA AAC-3′ and R 5′-GCG GAT TCT CTC AAT GTC-3′. The Probe sequence: 5-FAM-GCA TGT GGT TTA ATT CGA AGA TAC AC-TAMRA-3

Point mutations of *H. pylori 16S rRNA* gene were assessed. The assay was run on real time PCR ABI® step one using the following cycling conditions: The reaction was held for 10 min at a denaturing temperature of 95 °C, followed by 50 cycles of denaturing temperature of 95 °C for 10 s, an annealing temperature between 50 °C for 2 s and 55 °C for 1 s and extension temperature of 72 °C for 30 s. This was followed by a single probe melting cycle: 95 °C (ramp rate, 20 °C /s), 45 °C (ramp rate, 20 °C /s) and 80 °C (ramp rate, 0.5 °C/s).

The real time PCR assay produced a peak at a melting temperature: wild-type Tm of 63 °C and mutation point Tm of 58 °C.

3. Results

A total of 60 *H. pylori* infected patients were diagnosed by rapid urease test, and confirmed by detection of 16S rRNA gene in gastric biopsy (amplicon was detected at 110 bp) were enrolled in this study (Fig. 1). Forty patients (66.7%) were males and 20 patients (33.3%) were females. The patients' ages ranged between 17 and 76 years (49.93 ± 14.28 years), male/female ratio was 2:1.

Detection of the presence of mutant resistant genes conferring antimicrobial resistance for metronidazole (*rdxA* genes) was 25%, and for amoxicillin (*pbp1A*), it was 18.3%. Both of *rdxA* and *pbp1A* genes were detected by conventional PCR followed by sequencing (Table 1, Figs. 2 and 3)

Gene mutation in clarithromycin and tetracycline were tested by Real Time PCR. Resistance gene *23S rRNA* mutation type *A2143G* was detected in (6.7%) regarding clarithromycin while tetracycline showed only one strain (1.7%) having *16S rRNA* gene mutation (Table 1 Fig. 4).

Table 1
Distribution of antimicrobial resistance of *H. pylori* strains in gastric biopsy specimens from 60*H. pylori* infected patients.

Rate of resistance	*H. pylori* infected patients (n = 60)	
	Number	%
Metronidazole		
Detected mutation	15	25.0
Amoxicillin		
Detected mutation	11	18.3
Clarithromycin		
Wild	56	93.3
Mutant	4	6.7
Tetracycline		
Wild	59	98.3
Mutant	1	1.7

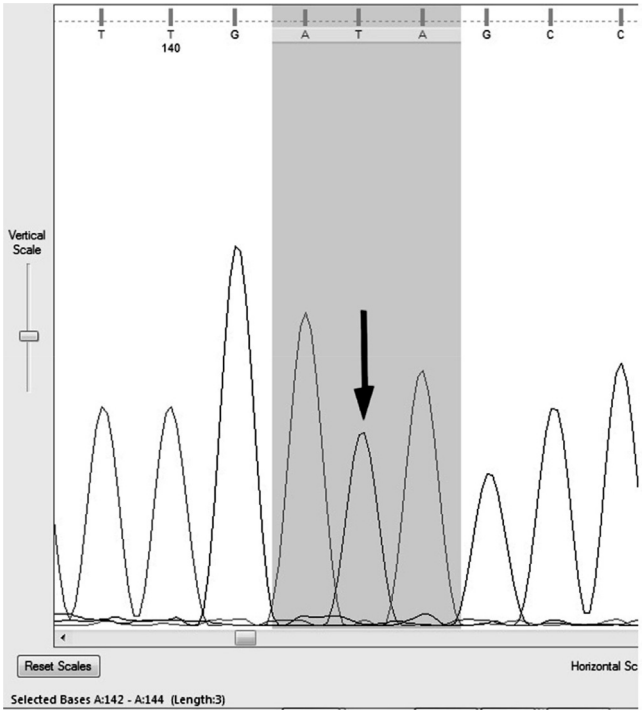

Fig. 2. DNA sequence analysis of PCR product of metronidazole *rdxA* gene from gastric biopsy. Diagram showed the wild type (A**C**A) substituted by the mutant type (A**T**A).

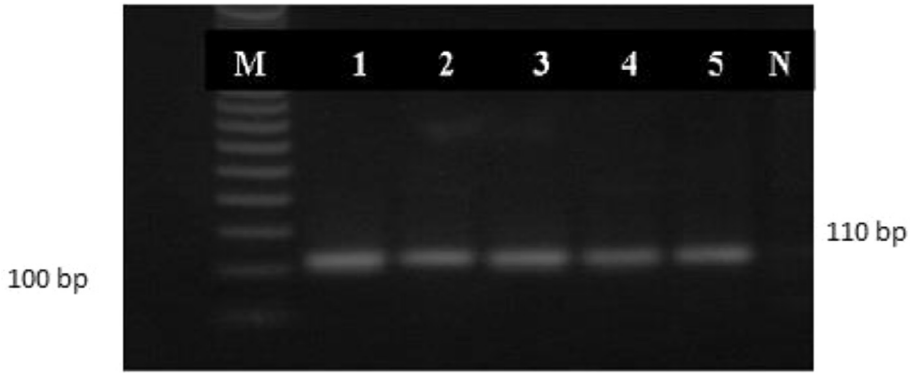

Fig. 1. Agarose gel electrophoresis of PCR products of *H. pylori 16S rRNA* from gastric biopsy. The 110 bp fragment was detected in samples 1-5(Lanes 1-5). M: molecular weight marker (ladder 50 bp). Lane N negative control.

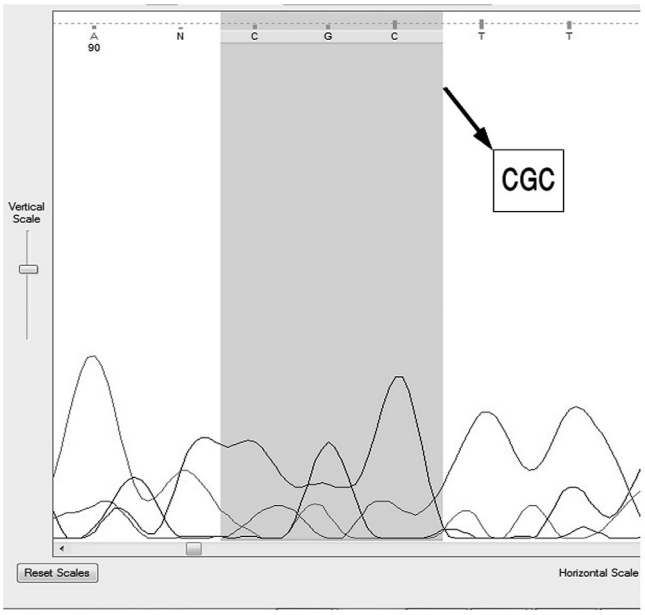

Fig. 3. Sequence analysis of PCR products of amoxicillin *pbp1A* positive gene from gastric biopsy. Diagram showed the wild type (G**C**G) substituted by the mutant type (**CG**C).

Combined resistance to metronidazole and amoxicillin (11.6%) was most frequently found, followed by metronidazole and clarithromycin (5%), while patterns of clarithromycin and amoxicillin, metronidazole, clarithromycin and amoxicillin were both 1.6% (Table 2).

4. Statistical analysis

Results are expressed as number (%). Statistical Package for Social Sciences (SPSS) computer program (version 19 windows) was used for data analysis. P value $\leq$0.05 was considered significant and <0.01 was considered highly significant.

5. Discussion

H. pylori infection is considered as one of the most frequent bacterial infections of the digestive system in the world [7]. *H. pylori* management in the clinical practice remains a challenge for the physicians. The choice of appropriate antibiotics is crucial in the success of treatment and recovery from *H. pylori*-related diseases. There are strong data showing that eradication of *H. pylori* infection reduces the risk of peptic ulcers, dyspepsia, and likely gastric cancer, if treated early in its natural history. However, the relative risk reduction of each complication varies [16].

Usually, the disease has chronic features unless it is treated by the combination of two proper antimicrobials; clarithromycin and metronidazole or amoxicillin, with proton-pump inhibitor or H2 receptor antagonist [17].

The current treatment of *H. pylori* is empirical. Therefore, the goal in designing a treatment regimen should focus on a strategy which results in a cure rate approaching 100% [18]. Eradication failure is concerning at the present time, it is due to a pre-existing antimicrobial resistant *H. pylori* strain or emergence of a new resistant strain from a susceptible ancestor. As reported worldwide, the successful eradication attempts are inversely correlated with antimicrobial resistance rates [19–21].

The main antimicrobial resistance mechanism is point mutations [22]. Real-time PCR has been used to successfully determine

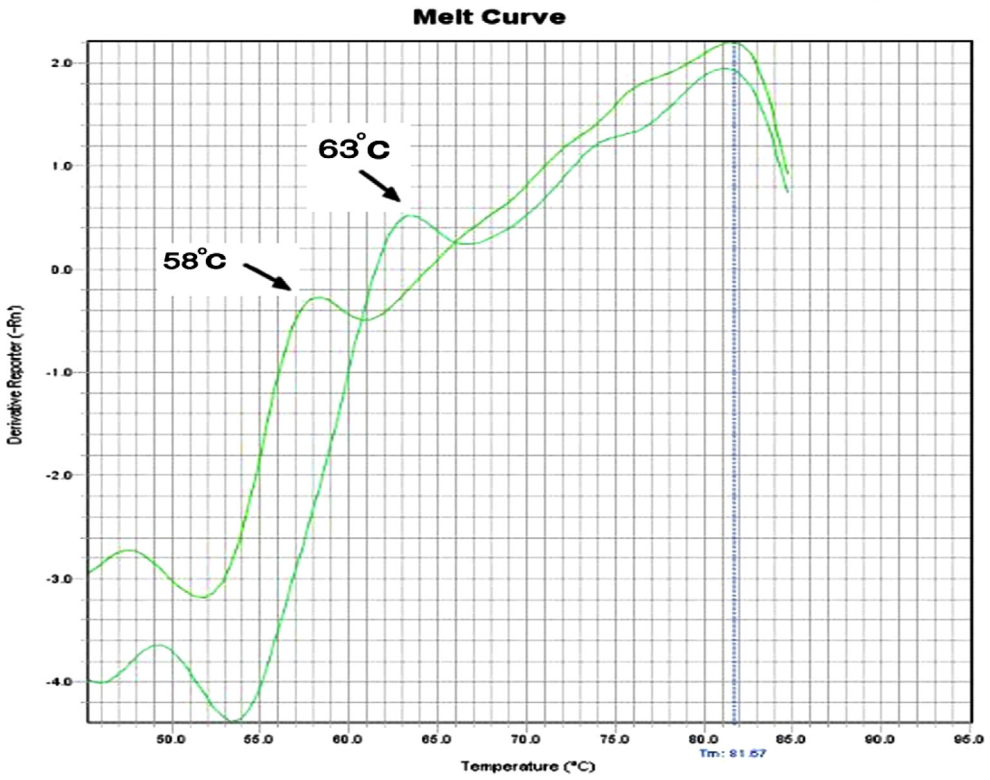

Fig. 4. Melting curve shows detection of *16S rRNA* mutation gene of tetracycline resistance from gastric biopsy. Diagram shows wild type at Tm 63 °C and mutant type at Tm 58 °C.

Table 2
Resistance rates of *H. pylori* to combined therapeutic agents in 60 patients infected *with H. pylori.*

Combined rate of resistance	*H. pylori* infected patients (n = 60)	
	Number	%
Metronidazole and Amoxicillin	7	11.6
Metronidazole and Clarithromycin	3	5
Clarithromycin and Amoxicillin	1	1.6
Metronidazole, Clarithromycin and Amoxicillin	1	1.6

H. pylori susceptibility to clarithromycin [23]. Another advantage of PCR is the potential to gather complete antimicrobial resistance data in patients infected with multiple strains of *H. pylori.*

Among the studied *H. pylori* gastric biopsy specimens, we reported considerable resistance against metronidazole (25%) and amoxicillin (18.3%) using conventional PCR assay followed by sequencing of DNA fragments of *rdxA* and *Pbp1A* genes respectively; and resistance rate to clarithromycin (6.7%) and little resistance to tetracycline using real time PCR for detection of *23S rRNA* and *16S rRNA* mutation respectively.

The prevalence of bacterial resistance varies in different geographic areas and appears to be increasing with time in many countries. It could be related to drug consumption, unregulated and extensive use of antibiotics [24]. Worldwide studies from African countries, suggested that *H. pylori* antimicrobial resistance rates is 17.2% for clarithromycin, 26.7% for metronidazole, 11.2% for amoxicillin and 16.2% for tetracycline [25,26].

Metronidazole is used as an alternative to clarithromycin and amoxicillin when a patient has an allergy or resistance to these two drugs [27]. We could detect its *rdxA* gene mutation in 25% of the studied *H. pylori* strains. Such resistance rate favors its use in first line therapy. Our result was comparable with that from England [28]; higher than that from the United States [29]; lower than reports from Tunisia (51.3%) [30] and Turkey (73%) [31]. Two previous Egyptian studies showed wide variation in frequencies of metronidazole resistance rates; Zaki et al. [26] suggested lower rate (12.8%) of resistance to metronidazole, while Fathi et al., [32] reported that only 25% of isolates were negative for *rdxA* gene by PCR, although100% of cases were phenotypically resistant to metronidazole by disc diffusion and E-test methods. The high resistance rate in the previous study maybe due to its use for gynecological, dental and parasitic related infectious diseases.

The prevalence of amoxicillin and tetracycline resistant rates has fortunately remained low worldwide [33]. Amoxicillin is used in combination with standard *H. pylori* therapy, the *pbp1A* gene associated with amoxicillin resistance among the studied *H. pylori* strains was detected in 18.3%, which was as good as that obtained by a previous Egyptian study [26], and a study in South East Asia by Kumala and Rani [34]. Resistance to amoxicillin has been shown to be negligible (0 to <2%) in European countries, such as Germany and the Netherlands [35,36]. On the other hand, *H. pylori* resistance rates to amoxicillin were 100%, 38%, and 32,8% in Nigeria [37], India [38] and Brazil [39] respectively. However, it has been suggested that *H. pylori* resistance to amoxicillin does not reduce treatment efficacy [40].

In the current study, the mutation sites of *16S rRNA* gene associated with tetracycline resistance were detected in 1.7% of *H. pylori* strains. The present data were matched with those recorded by Peretz et al. [7] and Ontsira et al. [41] who found that tetracycline resistance was seen in 2.3% and 2.5%; respectively. Comparable results had been reported by Shiota et al. [29] who found tetracycline resistance in 0.74% of the isolates. Rasheed et al. [31] could not detect neither amoxicillin nor tetracycline resistance in their study.

Since amoxicillin and tetracycline resistance are rare worldwide and bacterial resistance to metronidazole does not significantly affect eradication rates [42], the main concern is about clarithromycin which is used as an essential component of standard triple therapy for *H. pylori* [27].

The clarithromycin resistance is the major problem of treatment failure of *H. pylori* infection in many countries. Prevalence of primary clarithromycin resistance is increasing worldwide. Three main point mutations in *23S rRNA* at *A2143G*, *A2142G*, *A2142C* of domain V are responsible for more than 90% of clarithromycin resistance cases, and the most common is A-G transitions at position2143 (*A2143G*) [43].

In the current study mutation site of *23S rRNA* associated with clarithromycin resistance was determined at *A2143G* in 6.7% of *H. pylori* gastric biopsies which was slightly higher than previous Egyptian study (4%) by Sherif et al. [3] and it was comparable with Elviss et al. [44] in North Wales where the mutation site (A2143G) is the most common point of mutation site among the Italian population as it is associated with a higher eradication failure rate [45]. In areas of low resistance, treatments containing clarithromycin are recommended as a first-line empirical treatment [46]. None of our *H. pylori* strains had point mutation in *A2142G*. Acosta et al. [47] reported that the A2143G mutation was more frequent than the other mutation *A2142G*, and both were the most frequent mutations.

The clarithromycin resistance of *H. pylori* was significantly different between three geographical areas (17.5% in Europe, 18.9% in Asia and 29.3% in America). Among Asian countries, the high resistance rate of 84.9% and 40.7% was detected in China and Japan, respectively [25,48]. The variability of clarithromycin resistance seen in different regions emphasizes the need to examine resistance rates in each geographic area to better guide treatment regimens [49].

In the current study, the overall combined resistance rates were 11.6% for metronidazole and amoxicillin, 5% for metronidazole and clarithromycin and 1.6% for metronidazole, amoxicillin and clarithromycin. Elviss et al. [44] showed combined resistance for metronidazole and clarithromycin in 3.5% of their studied cases. A recent study by Phan et al. [50] showed that combined metronidazole and clarithromycin resistance was present in 15.2% and the triple resistance was present in 15.2%. A study by Picoli et al. [51] found that combined resistance to clarithromycin and amoxicillin was present in 18% of cases. Boltin et al. [52] showed that dual resistance to clarithromycin and metronidazole was seen in 39.9% which is much higher than our data.

Although clarithromycin resistance rate has reached more than 20% in many countries and regions worldwide, of which consensus guidelines recommended its removal from empirical triple therapy commonly used as a first line of treatment of *H. pylori* [53], our results suggested that the use of such therapy is still possible. Monitoring of anti-microbial susceptibility to *H. pylori* can be achieved through molecular methods which provide an attractive alternative to conventional culture methods. The use of molecular methods may allow the establishment of designed plans of *H. pylori* antimicrobial therapy that allows successful eradication and reduction of *H. pylori* associated disease and complications [54].

Although our data nearly have not crossed the 15–20% threshold for *H. pylori* antimicrobial resistance, there is a critical need to determine the current rates of such resistance. Such a determination would not only facilitate the selection of appropriate treatment regimens but also serve as a potential basis for transitioning to individualized analysis of antibiotic resistance prior to definitive treatment [49].

6. Conclusion

In conclusion, data concerning antimicrobial resistance genes play an important role in empiric treatment of *H. pylori* infection.

According to our results, *H. pylori* resistance to metronidazole and amoxicillin was relatively high. Clarithromycin is still a good option for first line anti-*H. pylori* treatment. Combined resistant strains are emerging and may have an effect on the combination therapy. In addition, constructing of an *H. pylori* resistance map using actual data from different regions of the world is crucial for the development. of appropriate eradication treatment alternatives. Without new treatment alternatives, we believe that *H. pylori* eradication may become an important problem in our country in the future. The increasing antimicrobial resistance of *H. pylori* and the emergence of multidrug-resistant strains are important issues that require the identification of new, more effective treatment alternatives.

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