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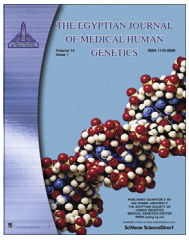

ORIGINAL ARTICLE

Genotypic and phenotypic patterns of antimicrobial susceptibility of *Helicobacter pylori* strains among Egyptian patients

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Abstract *Helicobacter pylori (H. pylori)* is currently recognized as one of the most common chronic bacterial infections worldwide. Eradication of bacteria is effective in healing peptic ulcers, preventing ulcer relapses, and potentially decreasing the risk of progression to gastric carcinoma. For successful eradication of bacteria, it is imperative that the clinician be aware of the current antimicrobial susceptibility profiles of isolates within the region. Therefore, the aim of this study is to compare the phenotypic and genotypic patterns of antibiotics' susceptibility to *H.* pylori strains among Egyptian patients.

60 symptomatic cases were enrolled. *H. pylori* infection was diagnosed by upper endoscopy as well as biopsy. Antimicrobial susceptibility to *H. pylori* strains was assessed in all subjects by disc diffusion and Ellipsometer testing (E-testing) methods. Further molecular characterization of genes

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[1] Author contributions: Marwa Saad Fathi and Runia Fouad EL-Folly designed the research; Marwa Saad Fathi, Rania Ahmed Hassan and Mohammed Ezz El-Arab performed the research. Marwa Saad Fathi and Rania Ahmed Hassan contributed analytic tools; analysed the data, Marwa Saad Fathi, Runia Fouad EL-Folly& Rania Ahmed Hassan wrote the paper.

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encoding antimicrobial resistance of isolated strains was done by the polymerase chain reaction (PCR).

For metronidazole and ciprofloxacin, we compared the phenotypic and genotypic patterns of resistance as detected by PCR amplification of the resistance genes. Resistance rates by E-test were 100% and 25% for metronidazole and ciprofloxacin respectively from 16 isolated *H. pylori* strains.

Improving the knowledge of resistance mechanisms, the elaboration of rational and efficacious associations for the treatment *H. pylori* infection are of high importance especially in determining the therapeutic outcome. Further progress should ultimately focus on the establishment of a cheap, feasible and reliable laboratory test to predict the outcome of a therapeutic scheme.

1. Introduction

Helicobacter pylori (*H. pylori*) infection, which affects half of the world's population, is responsible for gastritis [1], peptic ulcers [2,3] and gastric mucosa-associated lymphoid tissue lymphoma [4], and is a major risk factor for the development of gastric adenocarcinoma [5]. Eradication treatment of *H. pylori* infection usually consists of various combinations of drugs. Most commonly, an acid suppressor (usually a proton pump inhibitor) or a histamine receptor (H2-receptor) antagonist (e.g. ranitidine) is prescribed in combination with two antibiotics usually amoxicillin, metronidazole or clarithromycin. The combination of two antibiotics can increase the success of eradication therapy and decrease the possibility of secondary antibiotic resistance [6,7].

Antibiotic resistance in *H. pylori* is the major cause of eradication failure. Growing resistance often parallels the patterns of antibiotic consumption, and may vary within patient groups according to the geographic region, patient age and sex, type of disease, birthplace and the presence of other infections. The geographic map and the process of primary *H. pylori* resistance are clinically important, and should be considered when choosing eradication regimens, as is constant monitoring at both national and global level in an attempt to reach the recently recommended goal of eradicating more than 95% of resistant cases [8]. Different mechanisms of resistance to clarithromycin, metronidazole, quinolones, amoxicillin and tetracycline are accurately detailed (point mutations, redox intracellular potential, pump efflux systems, membrane permeability) on the basis of the most recent data available from the literature [9].

The prevalence of clarithromycin, metronidazole and amoxicillin resistance varies between countries and is highest for metronidazole [10]. Resistance to tetracycline and ciprofloxacin has been reported by several studies but yet appears uncommon [11,12].

The activation of metronidazole is mediated by the pyruvate: ferredoxin oxidoreductase complex. For example, this function in *H. pylori* might be fulfilled by the electron carriers, RdxA (HP0954), an oxygen-insensitive NADPH nitroreductase, FrxA (HP0642), a NAD(P)H-flavin oxidoreductase, ferredoxin (FdxA, HP0277), flavodoxin (FldA, HP1161), pyruvate: ferredoxin oxidoreductase (PorD, HP1109) and 2-oxoglutarate ferredoxin oxidoreductase (OorD, HP0588). Inactivation of the genes involved in some of these systems has been found to be linked to metronidazole resistance [13].

There are several problems with antimicrobial susceptibility testing of *H. pylori* [14,15]. Agar or broth dilution methods are difficult to perform routinely [16], thus, disc diffusion testing is often used because it is simple, easy to perform, and economical [17]. However, the E-test has proved to be an accurate method for assaying the susceptibility of fastidious organisms, including *H. pylori*, to antibiotics. The E-test has a more stable pattern of antibiotic release and has been found to tolerate prolonged incubation [18]. This is the main reason why the E-test rather than the disc diffusion method, has been recommended for *H. pylori* [19].

With the increasing frequency of clarithromycin resistance among *H. pylori* strains, there is rising concern about the potential decline in the eradication rate of this infection [20,21]. There is therefore an urgent need to introduce other treatment options. Fluoroquinolones, such as ciprofloxacin (CIP), levofloxacin (LVX) and moxifloxacin (MOX), have been evaluated as an alternative to standard antibiotics against *H. pylori* [6,7].

Some studies have shown good results when using fluoroquinolone based triple therapies for *H. pylori* eradication. In a German study, a 7 day course including LVX in patients with persistent *H. pylori* infection resulted in eradication rates of greater than 85% [22]. Similarly, an Italian study clarified that *H. pylori* eradication was achieved in 90% of patients treated with MOX, clarithromycin and lansoprazole [23]. However, the widespread use of fluoroquinolones for the treatment of *H. pylori* infection has led to an increase in its resistance rate in some areas, leading to unacceptably low eradication rates [10]. Several studies have shown that LVX based therapies are not superior to the traditional quadruple therapy or triple therapy in the treatment of *H. pylori* infection, especially in the case of resistant *H. pylori* strains [24,25].

On the other hand a Turkish study speculated that the low eradication rate with MOX containing treatment regimens may be due to the development of resistance to this quinolone [26]. The findings from all of these studies indicate that a regimen that is effective in one area may not be effective in another area, as antibiotic resistant rates for *H. pylori* may be different in different areas [27].

The mechanism of action of fluoroquinolones is via inhibition of deoxyribonucleic acid (DNA) gyrase and topoisomerase, which control and modify the topological state of DNA in cells. Fluoroquinolone then interferes with bacterial DNA replication. Both DNA gyrase and topoisomerase are composed of two A and two B subunits, encoded by the gyrA and gyrB genes for DNA gyrase and the parC and parE genes for topoisomerase. The mechanism of fluoroquinolone resistance in *H. pylori* has been found to be linked to mutations in the quinolone resistance determining regions (QRDRs) of the gyrA gene. Mutations in the gyrB gene have also been re-

ported in LVX resistant strains isolated in Japan, but these often occurred along with gyrA mutations [8].

In vitro susceptibility testing for *H. pylori* can be performed either by phenotypic or genotypic methods. Phenotypic testing is challenging because the organism grows slowly even under optimal culture conditions. Owing to these difficulties and because antibiotic resistance in this microorganism is essentially due to point mutations, genotypic methods are an alternative to the phenotypic methods. Moreover, these methods offer the advantage of testing directly from biopsy material, allowing a faster response.

2. Aim of the study

The aim of this study is to compare the phenotypic and genotypic patterns of antibiotics' susceptibility to *H.* pylori strains among Egyptian patients in order to attain a clinical utility from such patterns.

3. Subjects and methods

3.1. Study design and sampling

This cross-sectional study was conducted on 60 cases with epigastric pain and/or vomiting attended to the outpatient clinics and/or the endoscopy centre of Ahmed Maher Hospital during the period from May 2011 to January 2012. The sample size was calculated by Epi Info program (version 6.0) at 95% Confidence Limit, Power of the Test is 80% and Alpha Set at 0.05 (Type I error).

3.1.1. Inclusion criterion

Patients with epigastric pain and/or vomiting.

3.1.2. Exclusion criteria

Patients who refused to be enrolled in the study, patients who previously underwent sclerotherapy or band ligation of oesophageal varices (EV), patients taking drugs for primary prophylaxis of variceal bleeding, patients with chronic liver disease and/or hepatocellular carcinoma, patients with portal, splenic or hepatic vein thrombosis and patients with severe cardiac, chest or renal disease.

3.2. Tools of the study

All patients were subjected to a complete clinical evaluation and laboratory investigations: including: complete blood count (CBC), liver profile and hepatitis markers: Hepatitis B surface antigen (HBs Ag) and Hepatitis C virus antibody (HCV Ab) using the third generation enzyme-linked immunosorbent assay (ELISA) test **(Franciscus, 2002 and**[29]). Abdominal ultrasonography was done using Toshiba "Just vision" real-time scanner instrument with a 3.5 MHz convex transducer (after an overnight fasting) with stress on: liver size and liver echogenicity. Upper gastrointestinal (GI) endoscopy and endoscopic guided biopsy were done to exclude the presence of varices in addition to any relevant upper GI masses and to evaluate the degree of gastritis or the presence of ulcers using Pentax EG-3440 videoscope system. The endoscopic study was performed by the same examiner in all patients to avoid interobserver variability [30]. Gastric biopsies were collected on phosphate buffered saline (PBS) in sterile eppendorf tubes. Some patients had received *H. pylori* eradication therapy before, but none of the patients had received MOX or CIP based therapy [30].

3.3. Identification and storage of H. pylori strains

The biopsy specimens were cultured on Colombia blood agar (BBL Microbiology Systems, Cockeysville, MD, USA), supplemented with 8% defibrinated sheep blood, and incubated for 5–7 days under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) [30].

Clinical isolates were identified as *H. pylori* based on morphology and positive biochemical tests for catalase, oxidase, and confirmed by the rapid urease production test and gram staining.

Sixteen pure isolates were identified as *H. pylori*. They were stored at −80 °C in brain–heart infusion broth (BHI, Difco Laboratory, Detroit, MI, USA) supplemented with 30% glycerol.

3.4. Antimicrobial susceptibility to H. pylori strains

3.4.1. Antimicrobial susceptibility testing by disc diffusion method

For in vitro susceptibility testing of the *H. pylori* strains, a suspension equal to the McFarland tube No. 3 was prepared for each isolate. We used only one colony from each isolate for the analysis. Brain heart infusion broth (Merck, Germany) plates, supplemented with sheep red blood cells (RBCs) were inoculated by confluent swabbing of the surface with the adjusted inoculum suspensions.

The antimicrobial discs (Mast Diagnostics; Mast Group Limited, Mereyside, UK) for the antibiotics amoxicillin, metronidazole, tetracycline, ciprofloxacin, and clarithromycin, were aseptically placed onto the dried surface of inoculated Muller Hinton's agar plates supplemented with sheep RBCs. The plates were then incubated at 37 °C under microaerobic conditions [31]. The zones of inhibition were read after 24–48 h of incubation and the susceptibility results were recorded as resistant on the basis of the Clinical and Laboratory Standards Institute (CLSI) guidelines and the manufacturer's instructions [28,31].

3.4.2. Determination of the minimum inhibitory concentrations (MIC)

The E-test strips (Liofilchem, Italy) for the antibiotics amoxicillin, metronidazole, tetracycline, ciprofloxacin, and clarithromycin were aseptically placed onto the dried surface of inoculated agar plates. The plates were then incubated at 37 °C under microaerobic conditions. The minimum inhibitory concentrations (MICs) were read after 48–72 h of incubation on the basis of the intersection of the elliptical zone of growth inhibition using the MIC scale on the E-test strip, and the susceptibility results were recorded as resistant according to the recommendations of CLSI (Pennsylvania, USA) and the manufacturer's instructions [28,31].

A polymerase chain reaction (PCR) was performed for the 16 isolates to detect Ure C gene (a 1335 base pair (bp) long open reading frame upstream from the urease structural genes (ureAB) of *H.* pylori whose product is important for cell growth) for confirmation of the identification of *H pylori*. An-

Table 1 Descriptive data of positive findings of the studied cases.

Variables			Studied cases no. = 60	
			No.	%
Age (years)	Mean ± SD		46.7 ± 8.8	
	Range		5.0–60.0	
Sex	•Female		22	36.7%
	•Male		38	63.3%
HCV-Antibodies			18	30.0%
Clinical symptoms and signs	Nausea		22	36.7%
	Haematemesis		6	10.0%
	Abdominal distension		22	36.7%
	Epigastric pain		52	86.7%
Laboratory investigations	Complete blood count	Haemoglobin	11.9 ± 1.9	
		WBCs	4.8 ± 1.5	
		Platelets	178.0 ± 26.0	
	Liver enzymes	ALT	29.7 ± 29.1	
		AST	36.9 ± 31.9	
Abdominal ultrasound	Liver size	Average	46	76.7%
		Enlarged	14	23.3%
	Liver echogenicity	Coarse	12	20.0%
		Bright	12	20.0%
		Homogenous	24	40.0%
		Diffuse	12	20.0%
Upper endoscopy	Duodenitis		28	46.7%
	Antral erosions		26	43.3%
	Both		6	10.0%
Grading	Grade 0		42	70.0%
	Grade I		4	6.7%
	Grade II		10	16.7%
	Grade III		4	6.7%
Culture	Positive		16	26.7%
	Negative		44	73.3%
Antibiotic disc diffusion sensitivity test	Tetracycline	Sensitive	6	37.5%
		Resistant	10	62.5%
	Clarithromycin	Sensitive	0	0%
		Resistant	16	100.0%
	Metronidazole	Sensitive	0	0%
		Resistant	16	100.0%
	Ciprofloxacin	Sensitive	14	87.5%
		Resistant	2	12.5%
	Amoxycillin	Sensitive	2	12.5%
		Resistant	14	87.5%
E-test	Clarithromycin	Intermediate	8	50.0%
		Resistant	8	50.0%
	Metronidazole	Sensitive	0	0%
		Resistant	16	100.0%
	Cirprofloxacin	Sensitive	12	75%
		Resistant	4	25%
	Amoxycillin	Resistant	16	100%
	Tetracycline	Sensitive	4	25%
		Resistant	12	75%
PCR	gyrA and gyrB genes	Present	16	100.0%
	Rdx gene	Present	12	75.0%
		Absent	4	25.0%

other PCR was performed for the confirmed strains for detection of the DNA gyrase (gyrA and gyrB) genes responsible for resistance to fluoroquinolones and also the rdx gene responsible for the resistance to metronidazole.

3.5. Polymerase chain reaction (PCR) procedure

3.5.1. Extraction of DNA

1.5 ml of broth culture of each isolate was taken for extraction of DNA. Extraction was performed using QIAamp® DNA MINI KIT (QIAGEN, USA) (Catalog No. 51304) according to the manufacturer's instructions.

3.5.2. Amplification of the extracted DNA

Amplification of the eluted DNA was performed in a 50 µl reaction volume for each of the four detected genes. Each PCR mix consisted of 25 µl of ***2x QIAGEN HotStar Taq® Master Mix*** Containing HotStarTaq® DNA Polymerase, PCR buffer (with 3 mM $MgCl_2$), and 400 µM each dNTP + 10 µl of the eluted DNA + 1 µl of each of the 2 primers (50 pM) of the gene to be detected + 13 µl distilled water.

Primers were synthesized by (Roche, Germany).

Amplification of DNA was performed in a thermal cycler (thermo PxE 0.21, England).

3.5.3. Detection of the ureC gene

It was detected in the reaction mix described above using the following primers [38]:

5′-AAGCTTTTAGGGGTGTTAGGGGTTT-3′ and R 5′-AAGCTTATTTCTAACGC-3′. Thermocycling conditions were 35 cycles at 94° C for 1 min, 55° C for 1 min, and 72° C for 1 min, with a final extension step of 72 °C for 10 min., which amplifies a **295**-bp amplicon.

3.5.4. Detection of the rdxA gene

It was detected in the reaction mix described above using the following primers [38]:

rdxA1 (5′-AATTTGAGCATGGGGCGA-3′) and rdxA2 (5′-AAACGCTTGAAAACACCCT-3′)

Thermocycling conditions were 35 cycles at 94° C for 1 min, 55° C for 1 min, and 72° C for 1 min, with a final extension step of 72 °C for 10 min., which amplifies a **851**-bp amplicon.

3.5.5. Detection of the gyrA and gyrB genes

It was detected in the reaction mix described above using the following primers [8]:

Gyr APF (5′-AGCTTATTCCATGAGCGTGA-3′) and gyr APR (5′-TCAGGCCCTTTGACAAATTC-3′), gyrBPF (5′-CCCTAACGAAGCCAAAATCA-3′) and gyr BPR (5′-GGGCGCAAATAACGATAGAA-3′) that were designed to amplify a 582 bp and 465 bp amplicons, respectively.

Table 2 Comparative assessment between culture positive and culture negative by upper endoscopy.

Variables			Culture positive no. = 16	Culture negative no = 44	*P*	Sig.
Age	Mean ± SD		50.9 ± 7.5	45.2 ± 8.9	.027‡	S
Sex	Male		10 (62.5%)	28 (63.6%)	.936*	NS
	Female		6 (37.5%)	16 (36.4%)		
HCV-antibodies			2 (12.5%)	16 (36.4%)	.112**	NS
Clinical symptoms and signs	Nausea		6 (37.p 5%)	16 (36.4%)	.936*	NS
	Haematemesis		0 (.0%)	6 (13.6%)	.179**	NS
	Abdominal distension		8 (50.0%)	14 (31.8%)	.196*	NS
	Epigastric pain		14 (87.5%)	38 (86.4%)	1.00**	NS
Abdominal US	Liver size	Average	12 (75.0%)	34 (77.3%)	1.00**	NS
		Enlarged	4 (25.0%)	10 (22.7%)		
	Liver echo	Coarse	0 (.0%)	12 (27.3%)	.098**	NS
		Bright	4 (25.0%)	8 (18.2%)		
		Homogenous	8 (50.0%)	16 (36.4)		
		Diffuse	4 (25.0%)	8 (18.2%)		
Upper endoscopy	Duodenitis		6 (37.5%)	22 (50.0%)	.630**	NS
	Antral erosions		8 (50.0%)	18 (40.9%)		
	Both		2 (12.5%)	4 (9.1%)		
Grading	Grade 0		0 (0.0%)	42 (95.5%)	.0001**	HS
	Grade I		2 (12.5%)	2 (4.5%)		
	Grade II		10 (62.5%)	0 (0.0%)		
	Grade III		4 (25.0%)	0 (0.0%)		

NS: Non significant.
S: Significant.
HS: Highly significant.
* Chi square test.
** Fisher's exact test.
‡ Student's *t* test.

Thermocycling conditions were 35 cycles at 94° C for 1 min, 53° C for 1 min, and 72° C for 1 min, with a final extension step of 72 °C for 10 min.

Identification of amplified products by gel electrophoresis was done as the following [32]:

The amplicons (2 μl) (added to a Loading buffer: bromophenol blue with sucrose) was analysed by electrophoresis on 2% agarose gel and ethidium bromide staining in tris–acetate-EDTA (TAE) running buffer. They were visualized on a UV transilluminator (365 wave length). Qiagen gel pilot 1 Kb plus. (cat No. 239045) was used as molecular weight ladder.

3.5.6. Data management and statistical analysis

The collected data were revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (SPSS 15.0.1 for windows; SPSS Inc, Chicago, IL, 2001). Quantitative non parametric variables are expressed as mean and standard deviation (SD), while non parametric were expressed as Median and Interquartile range (IQR). Qualitative variables are expressed as frequencies and percents. Student's *t* test and ANOVA test were used to compare a continuous variable between two and more than two study groups respectively. Chi square and Fisher's exact test were used to examine the relationship between categorical variables. Marginal homogeneity test was used to assess the statistical significance of the difference of a variable with multiple categories measured by two different methods for the same study group. *P*-value: level of significance ($P > 0.05$: Non significant (NS), $P < 0.05$: Significant (*S*) and $P < 0.01$: Highly significant (HS)).

4. Results

A total of 16 *H. pylori* strains were isolated from 60 patients included in this study, 10(62.5%) from males and 6(37.5%) from females. The demographic, clinical, laboratory, endoscopic examination and antibiotic sensitivity data of the 60 patients enrolled in the study are presented in Table 1. Thirty-eight of the cases were males (63.3%), the mean ± SD of

Table 3 Comparative assessment between different endoscopic findings.

Variables			Duodenitis no. = 28	Antral erosions no. = 26	Both no. = 6	*P*	Sig.
Age	Mean ± SD		46.07 ± 8.205	49.38 ± 7.803	38.33 ± 11.255	.016*	S
Sex	Male		14 (50.0%)	18 (69.2%)	6 (100.0%)	.055**	NS
	Female		14 (50.0%)	8 (30.8%)	0 (.0%)		
Clinical symptoms and signs	Nausea		10 (35.7%)	12 (46.2%)	0 (0.0%)	.100**	NS
	Haematemesis		6 (21.4%)	0 (0.0%)	0 (.0%)	.033**	S
	Abdominal distension		8 (28.6%)	10 (38.5%)	4 (66.7%)	.242**	NS
	Epigastric pain		26 (92.9%)	20 (76.9%)	6 (100.0%)	.187**	NS
Grading	Grade 0		22 (78.6%)	16 (61.5%)	4 (66.7%)	.028**	S
	Grade I		0.0%	4 (15.4%)	0 (.0%)		
	Grade II		621.4%	4 (15.4%)	0 (.0%)		
	Grade III		0.0%	2 (7.7%)	2 (33.3%)		
Culture	Positive		6 2(1.4%)	8 (30.8%)	2 (33.3%)	.630**	NS
	Negative		22 (78.6%)	18 (69.2%)	4 (66.7%)		
Disc diffusion	Tetracycline	Sensitive	4 (66.7%)	0 (.0%)	2 (100.0%)	.003**	HS
		Resistant	2 (33.3%)	8 (100.0%)	0 (.0%)		
	Clarithromycin	Resistant	6 (100.0%)	8 (100.0%)	2 (100.0%)	….….	….
	Metronidazole	Resistant	6 100.0%)	8 (100.0%)	2 (100.0%)	….	….
	Ciprofloxacin	Sensitive	6 (100.0%)	6 (75.0%)	2 (100.0%)	.600**	NS
		Resistant	0 (.0%)	2 (25.0%)	0 (.0%)		
	Amoxycillin	Sensitive	0 (.0%)	2 (25.0%)	0 (.0%)	.600**	NS
		Resistant	6 (100.0%)	6 (75.0%)	2 (100.0%)		
E-test	Clarithromycin	Intermediate	4 (66.7%)	4 (50.0%)	0 (.0%)	.347**	NS
		Resistant	2 (33.3%)	4 (50.0%)	2 (100.0%)		
	Metronidazole	Resistant	6 (100.0%)	8 (100.0%)	2 (100.0%)	….….	….
	Ciprofloxacin	Sensitive	2(50%)	0(0%)	2 (50%)		
		Resistant	6 (50%)	6 (50%)	0(0%)		NS
	Amoxycillin	Resistant	8 (100%)	4 (100%)	4 (100%)		NS
	Tetracycline	Resistant	6 (100%)	3(100%)	3 (100%)		NS
		Sensitive	2 (50%)	2 (50%)	———		NS
PCR	Ciprofloxacin	Present	6 (100.0%)	8 (100.0%)	2 (100.0%)	….….	….
	Metronidazole	Present	4 (66.7%)	6 (75.0%)	2 (100.0%)	1.00**	NS
		Absent	2 (33.3%)	2 (25.0%)	0 (.0%)		

NS: Non significant
S: Significant
HS: Highly significant
* ANOVA.
** Fisher's exact test; NA.

age was 46.7 ± 8.8 (ranging from 50–60 years), 18% had positive HCV-Antibodies, epigastric pain was the main clinical presentation of the enrolled cases (86.7%). Upper endoscopy revealed duodenitis, antral erosions or both (46.7%, 43.3%, and 10.0%, respectively), 70% was grade 0 of the pathological examination, 73.3% was culture negative. **We also compared between culture positive and culture negative by upper endoscopy** (Table 2). Table 3 **shows a comparative assessment between different endoscopic findings**. Table 4 **shows a comparative assessment between different pathological grades**.

Regarding antibiotic disc diffusion sensitivity test, the sensitivity was 37.5% in tetracycline 100.0% in clarithromycin, 0% in Metronidazole, 87.5% in Ciprofloxacin, 12.5% in Amoxycillin. While concerning E-test, Clarithromycin was intermediate in 50.0%, the sensitivity in Metronidazole was 0%, Cirprofloxacin in 75%, Tetracycline in 25%. Table 5 shows a non- significant difference between males and females regarding the resistance rates to all the tested antibiotics either by disc diffusion, E-test or the results of PCR.

For molecular characterization of our strains, we firstly confirmed the presence of bacterial DNA of *H pylori* by amplifying the Ure C gene (a house keeping gene) (Fig. 1) by PCR. Then, secondly, we investigated the presence of resistance genes of Fluoroquinolones (gyrA and gyrB genes) which were found in 100% of isolates (Fig. 3) and Metronidazole (rdx gene) which was found in 75.0% (Fig. 2). Furthermore our results revealed an agreement between results of antimicrobial susceptibility testing by disc diffusion and E testing of (A) Clarithromycin & (B) Metronidazole (Fig. 4).

The 16 tested isolates (100%) were resistant to metronidazole by E-testing. However the rdxA gene was detected in 12 isolates (75%). While 12 (75%) isolates were resistant to ciprofloxacin by E-testing, with the gyrA and gyrB genes detected in 16 (100%) of isolates (Table 6).

5. Discussion

In the present study we evaluated the sensitivity of sixteen *H. pylori* strains isolated from patients with gastric disorders to five different antibiotics (metronidazole, tetracycline, clarithromycin, amoxicillin and ciprofloxacin) using the disc diffusion method. MIC determination for metronidazole and clarithromycin was done by E-test to find the resistance pattern in these strains in our region.

For metronidazole and ciprofloxacin, we compared the phenotypic and genotypic patterns of resistance as detected by PCR amplification of the rdxA, gyrA and gyrB genes.

In this work, the resistance rate to metronidazole detected by E-test was 100% consistent with reports from developing countries that described a high level of resistance to metronidazole, which varies from 66.2% to 100% [33,34]. However reports from developed countries record lower rates of resistance to metronidazole where it has been reported that 15.8% to 40% of *H. pylori* strains were resistant to metronidazole [35–37]. This discrepancy in resistance rates to metronidazole between developing and developed countries could be attributed to the frequent use of the drug, which is commonly prescribed for other diseases, especially parasitic conditions, and periodontal or gynaecological infections. Moreover, the use or abuse of this inexpensive drug may contribute to the increased metronidazole resistance seen in developing countries.

As shown in Table 5 both male and female patients showed 100% resistance rates to metronidazole by E-test and a non significant difference in detection of resistance gene. This disagrees with Farshad et al. [19] and Kargar et al. [38] who reported a higher resistance rate in females that could be attributed to the use of this drug for treatment of gynaecologic infections. The non significant difference we report may be due

Table 4 Comparative assessment between different pathological grades.

Variables			Grade I no. = 2	Grade II no. = 10	Grade III no. = 4	*P*	Sig.
			N (%)	N (%)	N (%)		
Disc diffusion	Tetracycline	Sensitive	0 (.0%)	4 (40.0%)	2 (50.0%)	.610**	NS
		Resistant	2 (100.0%)	6 (60.0%)	2 (50.0%)		
	Clarithromycin	Resistant	2 (100.0%)	10 (100.0%)	4 (100.0%)	———	——
	Metronidazole	Resistant	2 (100.0%)	10 (100.0%)	4 (100.0%)	———	——
	Ciprofloxacin	Sensitive	0 (0%)	10 (100.0%)	4 (100.0%)	.008**	HS
		Resistant	2 (100.0%)	0 (.0%)	0 (0%)		
	Amoxycillin	Sensitive	0 (.0%)	2 (20.0%)	0 (0%)	1.00**	NS
		Resistant	2 (100.0%)	8 (80.0%)	4 (100.0%)		
E-test	Clarithromycin	Intermediate	0 (0%)	6 (60.0%)	2 (50.0%	.504**	NS
		Resistant	2 (100.0%)	4 (40.0%)	2 (50.0%)		
	Metronidazole	Resistant	2 (100.0%)	10 (100.0%)	4 (100.0%)	———	——
	Ciprofloxacin	Resistant	4 (25%)	0 (0%)	0(0%)	———	
		Sensitive	0 (0%)	10 (100%0	2 (50%)		
	Amoxycillin	Resistant	2 (100%)	10(100%)	4 (100%)		
	Tetracycline	Resistant	2 (100%)	10(100%)	0 (0%)		
		Sensitive	0(0%)	4 (100%)	0 (0%)		
PCR	gyrA and gyrB	Present	2 (100.0%)	10 (100.0%	4 (100.0%)	———	——
		Absent	0 (0%)	4 (40.0%)	0 (0%)		
	rdx gene	Present	2 (100.0%)	6 (60.0%)	4 (100.0%)	.258**	NS

NS: Non significant.
HS: Highly significant.
** Fisher's exact test.

Table 5 Rates of antibiotic resistance in *Helicobacter pylori* isolates in relation to patient gender.

Variables			Male no. = 10	Female no. = 6 no. = ...	*P*	Sig.
			N (%)	N (%)		
Disc diffusion	Tetracycline	Sensitive	2 (20.0%)	4 (66.7%)	.118**	NS
		Resistant	8 (80.0%)	2 (33.3%)		
	Clarithromycin	Resistant	10 (100.0%)	6 (100.0%)	———	——
	Metronidazole	Resistant	10 (100.0%)	6 (100.0%)	———	——
	Ciprofloxacin	Sensitive	8 (80.0%)	6 (100.0%)	.500**	NS
		Resistant	2 (20.0%)	0 (.0%)		
	Amoxycillin	Sensitive	0 (.0%)	2 (33.3%)	.125**	NS
		Resistant	10 (100.0%)	4 (66.7%)		
E-test	Clarithromycin	Intermediate	4 (40.0%)	4 (66.7%)	.608**	NS
		Resistant	6 (60.0%)	2 (33.3%)		
	Metronidazole	Resistant	10 (100.0%)	6 (100.0%)	———	——
	Ciprofloxacin	Sensitive	5 (41%)	7(59%)		
		Resistant	2 (50%)	2(50%)		NS
	Amoxycillin	Resistant	12 (100%)	4 (100%)		NS
	Tetracycline	Resistant	6 (100%)	6(100%)		NS
		Sensitive	4(100%)	0(0%)		NS
PCR	Ciprofloxacin	Present	10 (100.0%)	6 (100.0%)	———	——
	Metronidazole	Present	8 (80.0%)	4 (66.7%)	.604**	NS
		Absent	2 (20.0%)	2 (33.3%)		

NS: Non significant.
** Fisher's exact test.

Table 6 Relationship between genotypic and E-test phenotypic pattern as regards sensitivity against metronidazole, relationship between genotypic and disc diffusion phenotypic pattern as regards sensitivity against metronidazole and ciprofloxacin.

Variables				rdx gene				*P*	Sig.
				Present = 12		Absent = 4			
				N	%	N	%		
E-test	Metronidazole	*	Sensitive	0	.0	0	.0	———	——
			Intermediate	0	.0	0	.0		
			Resistant	12	100.0	4	100.0		
		**	Sensitive	0	.0	0	.0	——	———
			Resistant	12	75.0	4	25.0		
	gyrA and gyrB genes								
	Ciprofloxacin	***	Sensitive	12	75	0	.0	——	———
			Resistant	4	25	0	.0		

to the narrow sample size and restriction of sampling from patients who routinely are candidates for upper gastroscopy.

Metronidazole is administered as a pro-drug that is activated by the reduction of the nitro group that is attached to an imidazole ring. Inactivation of an oxygen-insensitive NADPH nitroreductase (rdxA) may be responsible for metronidazole resistance. The activation of metronidazole in strictly anaerobic bacteria is mediated by the pyruvate: ferredoxin oxidoreductase complex. For example, this function in *H. pylori* might be fulfilled by the electron carriers, RdxA (HP0954), FrxA (HP0642), ferredoxin (FdxA, HP0277), flavodoxin (FldA, HP1161), pyruvate: ferredoxin oxidoreductase (PorD, HP1109) and 2-oxoglutarate ferredoxin oxidoreductase (OorD, HP0588) [13].

In this study we investigated the rdxA gene deletion as a factor of resistance to metronidazole. Table 4 shows that only 25% of isolates were negative for rdxA gene by PCR, although 100% of cases were phenotypically resistant to metronidazole by disc diffusion and E-test methods. This finding goes in alignment with Abdollahi et al. [13] who reported a 22.9% rate of rdxA gene deletion among their 35 tested resistant *H. pylori* strains. These results can be explained by the presence of mechanisms of rdxA inactivation rather than deletion, for example mutational inactivation which can be identified by further sequencing of the amplified DNA. Another mechanism that may be associated with rdxA gene inactivation is the insertion of a transposon called Mini-IS605 [39]. It has also been demonstrated that inactivation of other genes such as the *frxA* gene also confers metronidazole resistance, either alone or in association with the *rdxA* gene [40,41].

The present study revealed a high resistance rate to amoxicillin by E-test of 87.5% (Table 1). This rate agrees with

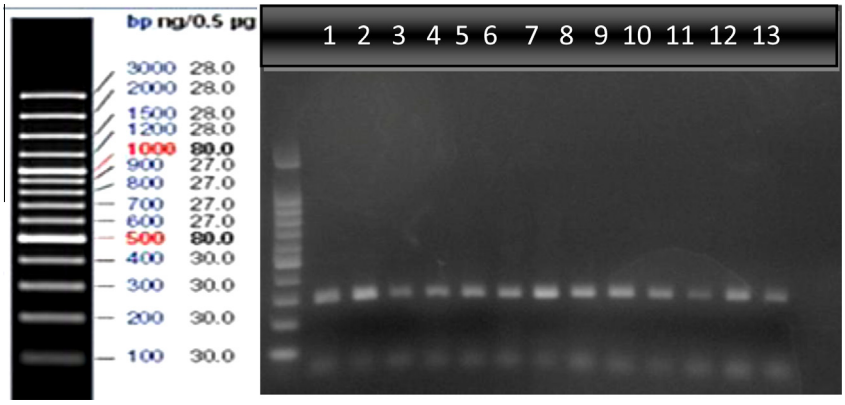

Figure 1 Gel electrophoresis showing PCR products (The ***ureC*** (*glmM*) gene **295**-bp amplicon) fragment size measured against GeneRuler™ 100 bp Plus DNA Ladder. Bands are seen in lanes from 1 to 13 (N.B three isolates were not photographed).

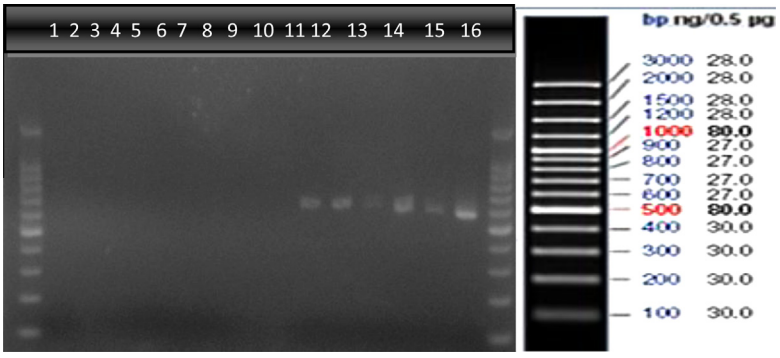

Figure 2 Gel electrophoresis showing PCR products (Metronidazole resistance gene {Rdx: 600 bp}) fragment size measured against GeneRuler™ 100 bp Plus DNA Ladder. Bands are seen in lanes 11 to 16.

Aboderin et al. [34] who reported 100% resistance in all the examined 32 isolates. In the same context, Okimoto and Murakami [42]; Godoy et al. [43] and Kumala and Rani [44] reported resistance rates to amoxicillin of 38%, 32.8% and 19.4%, respectively. In contrast to these rates, Boyanova et al. [37]; Malfeltheiner et al. [6] and John Albert et al. [45] reported that amoxicillin resistance was very rare or even non-existing. This wide variation in amoxicillin resistance rates reported from different countries could be attributed to the regional prescribing practice of the drug.

Clarithromycin is utilized in the recommended first line triple therapies against *H. pylori*. This study noticed a high resistance rate to clarithromycin of 100% by the disc diffusion method (Table 1), however only 50% (8 out of 16 isolates) were confirmed to be resistant to clarithromycin by E-test (Table 1). This rate agrees with that of Abadi et al. [46] who found that 45.2% of 197 *H. pylori* isolates are resistant to clarithromycin by the disc diffusion method. Furthermore, Ilie et al. [47] reported a resistance rate to clarithromycin of 32% of their tested isolates. In contrast, the rate of clarithromycin resistance reported in this study is much higher than that reported by Milani et al. [48] who found a rate of resistance of 14.3% (16 out of 112 *H. pylori* isolates). Similarly, Boyanova et al. [49] reported a lower rate of primary resistance to claithromycin of 17.9% of 519 *H pylori* strains. The high rate of clarithromycin resistance detected in this study is likely a consequence of an overuse of macrolides for the treatment of upper respiratory tract infections.

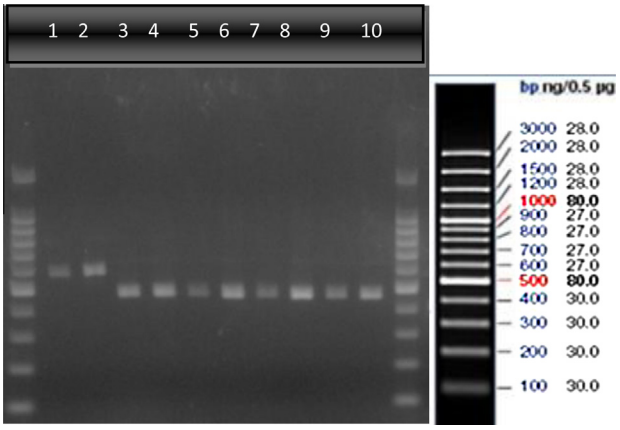

Figure 3 Gel electrophoresis showing PCR products (Fluoroquinolones resistance gene {gyr A: 582 bp, gyr B:465 bp}) fragment size measured against GeneRuler™ 100 bp Plus DNA Ladder . Gyr A bands are shown in lanes 1,2 while Gyr B bands are shown in lanes from 3 to 10.

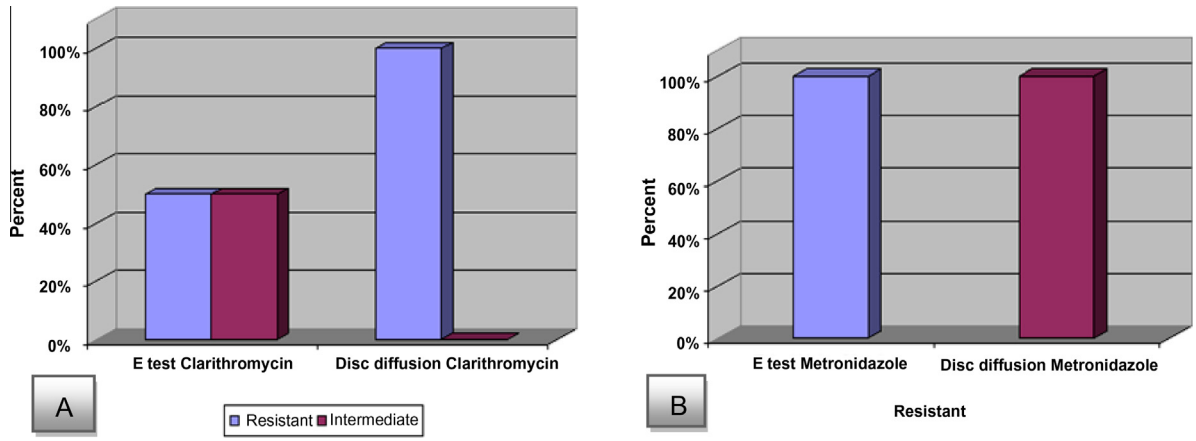

Figure 4 Agreement of results of antimicrobial susceptibility testing by disc diffusion & e Testing of (A) Clarithromycin & (B) Metronidazole.

Ciprofloxacin belongs to the fluoroquinolone group of antibiotics that are generally used as part of rescue therapy for treating *H. pylori* infections when first and second line therapies have failed [50]. Resistance to fluoroquinolones is generally very low (< 10%) worldwide [51]. In our study we found a low resistance rate for ciprofloxacin using the disc diffusion method (2 out of 16 isolates) 12.5% and 25% by E-testing (Tables 1 and 4) which matches with that detected by Boyanova et al. [49] that was 10.8% of 519 isolates, and with that of Chung et al. [52] who reported a resistance rate of 15.7% of 185 isolates detected by MIC. The rate reported in this study is lower than that detected by Abadi et al. [46] and Milani et al. [48] who reported resistance to ciprofloxacin in 34.5% of 197 isolates and in 33% of isolates (37 out of 112 isolates), respectively.

Further sequencing of the amplification products of the gyrA and gyrB genes should have been done for the detection of the point mutations related to resistance to ciprofloxacin. However, unfortunately it was not feasible to perform such technique.

In this study we detected 10 resistant strains to tetracycline out of the 16 tested isolates (62.5%) (Table 1), this rate is much higher than most reports. For example, Chung et al. [52] found only 1 resistant strain to tetracycline out of the 185 examined strains (0.5%). In the same context, Boyanova et al. [49] reported a rate of resistance of 4% for tetracycline. In the current study, a non significant difference was found between males and females regarding tetracycline resistance, and this lodges in controversy with Boyanova et al. [49] who allocated female sex as the only predictor of primary tetracycline resistance.

6. Conclusion

The present study elucidated the primary state of *H. pylori* isolates from Egyptian patients regarding their susceptibility to currently used anti-microbial drugs. It brings attention to establish a designed plan for antimicrobials prior to launching of treatment in order to accomplish better integration between microbiological assessment and clinical usage of antimicrobials on a broad scale and regular basis. This could contribute to further successful eradication of *H. pylori*, thus minimizing the risk of chronicity and consequently malignant transformation and sequential complications.

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