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ORIGINAL ARTICLE

GENETIC MUTATIONS AFFECTING THE FIRST LINE ERADICATION THERAPY OF *Helicobacter pylori*-INFECTED EGYPTIAN PATIENTS

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SUMMARY

Introduction: Several genetic mutations affect the first-line triple therapy for *Helicobacter pylori*. We aimed to study the most common genetic mutations affecting the metronidazole and clarithromycin therapy for *H. pylori*-infected Egyptian patients. **Patients and Methods**: In our study, we included 100 successive dyspeptic patients scheduled for diagnosis through upper gastroscopy at Cairo's University Hospital, Egypt. Gastric biopsies were tested for the presence of *H. pylori* by detection of the *16S rRNA* gene. Positive biopsies were further studied for the presence of the *rdxA* gene deletion by Polymerase Chain Reaction (PCR), while clarithromycin resistance was investigated by the presence of nucleotide substitutions within *H. pylori* 23S rRNA V domain using M*boII* and B*saI* to carry out a Restricted Fragment Length Polymorphism (RFLP) assay. **Results:** Among 70 *H. pylori* positive biopsies, the *rdxA* gene deletion was detected in 44/70 (62.9%) samples, while predominance of the A2142G mutations within the *H. pylori* 23S rRNA V domain was evidenced in 39/70 (55.7%) of the positive *H. pylori* cases. No statistically significant difference was found between the presence of gene mutations and different factors such as patients 'age, gender, geographic distribution, symptoms and endoscopic findings**. Conclusion**: Infection with mutated *H. pylori* strains is considerably high, a finding that imposes care in the use of the triple therapy to treat *H. pylori* in Egypt, since the guidelines recommend to abandon the standard triple therapy when the primary clarithromycin resistance rate is over 20%[1].

KEYWORDS: 16s rRNA; Metronidazole resistance; *rdxA* gene; Clarithromycin resistance.

INTRODUCTION

Eradication of *Helicobacter pylori* infection is challenging. The recommended first-line eradication therapy, especially in patients with peptic ulcer disease is based on the combination of a proton pump inhibitor, clarithromycin, together with amoxicillin or metronidazole[1]. However, recent data showed that the combination therapy has lost its efficacy due to the emergence of resistant strains. The rate of resistance is increasing worldwide with variations according to the geographic area[2].

The molecular mechanism of metronidazole resistance is mainly due to inactivation of an oxygen-insensitive NADPH nitroreductase (*rdxA*) responsible for metronidazole resistance as a result of a deletion in the *rdxA* encoding gene, and may also be boosted by mutations in the *frxA* gene that encodes a NAD(P)H-flavin oxidoreductase[3,4].

Furthermore, the major cause of clarithromycin resistance in *H. pylori* is the lack of binding of the macrolide to the 23S rRNA components of the bacterial ribosome due to modification of the target site by point mutations in the peptidyl transferase region of domain V of the 23S rRNA[5,6]. The A2142G and A2143G nucleotide substitutions are the most common mutations causing clarithromycin resistance worldwide[6,7].

In Egypt, the majority of clinicians prescribe the classical triple regimen composed of clarithromycin; metronidazole and a proton pump inhibitor for seven days as the first line therapy to treat *H. pylori* infections. The high failure rate of the first line triple therapy encourages the investigation of the prevalence and genetic background of clarithromycin and metronidazole resistance in *H. pylori*-infected patients.

PATIENTS AND METHODS

Patients and specimens

This study included 100 dyspeptic patients attended at the endoscopy

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unit of the Kaser Al Aini, Cairo's University Hospital, to undergo upper gastrointestinal (GI) endoscopy, from January 2013 to November 2013. The study included 54% males and 46% females, aged 21-60 years old (mean 44 +/- 11.1) and 53% of the studied patients lived in urban areas. The patients had not received the first-line triple therapy for *H. pylori* (clarithromycin, metronidazole and proton pump inhibitors), i.e., they were naive regarding all of the eradication therapy drugs so as to determine the primary resistance rate to clarithromycin and metronidazole. An informed consent was obtained from each patient.

Patients who were less than 30 years or more than 60 years old were excluded from the study, as well as patients with any contraindications to undergo upper GI endoscopy, or had been submitted to upper GI endoscopy for other reasons (e.g. cirrhotic patients with varices).

Upper GI endoscopy was conducted by means of an Olympus endoscope video, and 100 gastric biopsies from 100 patients were performed from the stomach corpus or antrum, placed in glycerol solution and kept at -80 ^{0}C and then sent to the clinical pathology department laboratory for further laboratory work up.

DNA extraction

DNA was extracted from gastric biopsies using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer recommendations.

16s rRNA-PCR

PCR was performed on extracted DNA targeting the *H. pylori* 16s *rRNA* gene (Hp16s). The following cycling conditions were used: 35 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec and an extension time of 72 °C for 5 min according to Secka *et al.* 2011[8]. All of the primers used in this study are listed in Table 1.

rdxA gene deletion-PCR

All of the *H. pylori*-positive samples for the 16s rRNA-PCR were submitted to the detection of metronidazole-resistance gene *rdxA* gene by PCR. By this method, the expected molecular weight of the PCR fragment for the wild type *rdxA* gene is 850 bp, and for the mutated allele 650 bp[3]. PCR reactions were carried out in 25 µL mixtures containing 12.5 µL of the master mix (QIAGEN, Hilden, Germany) 9.5 µL of sterile deionized water, 1 µL of the template DNA and 1 µL of each of the oligonucleotide primers. The thermos cycler conditions were as follows: an initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 55 °C, extension at 72 °C for 1 min. The final extension step was extended to 10 min at 72 °C[3].

Detection of 23S rRNA mutations by PCR-RFLP analysis

DNA amplification was carried out using *Taq* PCR Master Mix Kit supplied by (QIAGEN, Hilden, Germany) on the extracted DNA using the primers Hp23Sr6 (sense) and Hp23Sr7 (antisense). The amplified DNA product corresponded to the domain V of the *H. pylori* 23S rDNA. The cycling conditions were as follows; denaturation at 94 °C 5 min followed by 40 cycles at 94 °C for 30 sec; 60 °C for 30 sec; 72 °C for 30 sec and one final extension cycle at 72 °C for 7 min, in a total volume of 25 µL containing 1× PCR buffer, 200 µM dNTPs, 2.0 mM MgCl2, 1 µM of each oligonucleotide primer, 1.25 U Taq DNA according to Suzuki *et al*. 2013[9].

The amplified DNA fragments were digested with M*boII* and B*saI* (New England Biolabs) according to the manufacturer instructions. These restriction enzymes are able to discriminate mutations within the *H. pylori* domain V of the 23S rDNA at positions 2,142 and 2,143, respectively[9].

In all the PCR reactions a negative and a positive control were used corresponding to, sterile water and *H. pylori* positive gastric biopsies, respectively.

The amplified fragments were detected by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized using Gel Doc XR documentation system (Bio-Rad, Hercules, CA, USA).

Statistical analyses

Data were statistically described in terms of mean +/- standard deviation (+/- SD), median and range, or frequencies (number of cases) and percentages when appropriate. Comparison of quantitative variables between the study groups used the Mann–Whitney U test for independent samples. For comparison of qualitative variables, the Chi-square (X^2) test was performed. The Fisher's exact test was used when the expected frequency was less than 5. The agreement was calculated using the Kappa test. Sensitivity, specificity, positive predictive value, negative predictive value, and overall accuracy were calculated and *p*-values less than 0.05 were considered statistically significant. All the statistical calculations were performed using the SPSS software, Statistical Package for Social Sciences (IBM, NY, USA) version 15 for Windows.

Table 1
Sequence of primers used in this study

Gene	Primers '5-3'	Expected fragment (bp)	References
Hp1	CTGGAGAGACTAAGCCCTCC	109bp	[8]
Hp2	ATTACTGACGCTGATTGTGC		
rdxA1	AATTTGAGCATGGGGCAGA	850bp	[3]
rdxA2	GAAACGCTTGAAAACACCCCT		
Hp23Sr6	CACACAGGTAGATGAGATGAGTA	768bp	[9]
Hp23Sr7	CACACAGAACCACCGGATCACTA		

Hp: *H. pylori*

RESULTS

From the total of 100 extracted DNA samples, 70% were positive by the *H. pylori 16S rRNA* gene-PCR, yielding the 109 bp product. Demographic features of the Hp16S-positive patients are shown in Table 2, and the clinical features of Hp16S-positive patients as well as the endoscopic findings of Hp16S-positive patients are shown in Tables 3 and 4, respectively.

Among the seventy positive *H. pylori* samples, 44 (62.9 %) were also positive by the *rdxA gene*-PCR yielding a 650 bp product, whereas in 19 (27.1%) the *rdxA gene* was positive, but the amplification product had a molecular weight of 850 pb. The RFLP pattern showed that 39/70 (55.7%) samples had the A2142G mutation as shown in Figures 1 and 2. Moreover, none of the tested samples had the A2143G mutation.

DISCUSSION

The World Gastroenterology Organization (WGO) reported that the *H. pylori* prevalence in Egypt was 90% in adults. Regarding the Middle East, *H. pylori* prevalence ranges from 60-90% as documented by WGO[10]. The first line triple therapy regimen has proved to become inefficient worldwide, mainly as a result of the emergence of several genetic mutations reducing *H. pylori* susceptibility to clarithromycin and metronidazole.

H pylori is a relatively fastidious slowly growing microaerophilic bacterium a characteristic that makes the conventional culture method as well as the *vitro* antibiotic susceptibility tests (disk diffusion, agar dilution, and Epsilometer tests) time consuming, requiring 10-14 days to release a final report on the isolated *H. pylori* sensitivity. Molecular diagnostic techniques constitute attractive alternative methods to determine antibiotic susceptibilities with better accuracies and shorter turnaround time when performed directly on gastric biopsies, without culture[11].

Molecular assays for the detection of resistance to metronidazole are still controversial. A large number of mutations have been described in *rdxA, frxA and frxB* genes, but their true role in metronidazole resistance is not clear. In our study, the prevalence of *rdxA* gene mutation was 62.9%. Spreading of the *rdxA* gene mutation can be explained by the empiric use of metronidazole for the treatment of parasitic and anaerobic infections in Egypt. The drug dosage in *H. pylori* infections has contributed to the emergence of resistant mutants, but is not able to eradicate the organism, leading a selective pressure effect.

In another Egyptian study conducted by Sherif *et al.* (2004) the phenotypic metronidazole resistance was found in 100% of the 48 *H.*

Table 2
Demographic features of *H. pylori* positive patients

	PCR for *H. Pylori*				*p* value
	Positive (n=70)		Negative (n=30)		
Sex	N	%	N	%	
Male	40	57.1	14	46.7	
Female	30	42.9	16	53.3	0.4 NS
Residence	N	%	N	%	
Urban	36	51.4	17	56.7	
Rural	34	48.6	13	43.3	0.7 NS
Age years Mean ± SD	43.6 ±11.3		44.9 ±10.6		0.6 NS

NS: non-significant

Table 3
Clinical features of *H. pylori* positive patients

	PCR for *H. Pylori*				*p* value
	Positive (n=70)		Negative (n=30)		
	N	**%**	**N**	**%**	
Epigastric pain	61	87%	25	83%	0.8 NS
Vomiting	50	71.4%	21	70%	1.0 NS
Heartburn	36	51.4%	21	70%	0.1 NS
Regurgitation	23	32.9%	13	43.3%	0.4 NS
Bleeding	22	31.4%	8	26%	0.8 NS

NS: non-significant

Table 4
Endoscopic findings of *H. pylori* positive patients

	PCR for *H. Pylori*				*p* value
	Positive (n=70)		Negative (n=30)		
	N	**%**	**N**	**%**	
Diffuse gastritis	33	47.1%	9	30%	0.1 NS
Reflux esophagitis	23	32.9%	9	30%	0.8 NS
Bulb duodenitis	19	27.1%	4	13.3%	0.2 NS
Antral gastritis	10	14.3%	6	20%	0.6 NS
Duodenal ulcer	7	10%	0	0 %	0.1 NS
Gastric mass	5	7.1%	5	16.7%	0.2 NS
Esophageal varices or PHG	4	5.7%	2	6.7%	1.0 NS
Others	4	5.7%	2	6.7%	1.0 NS
Gastric ulcer	3	4.3%	2	6.7%	0.6 NS
Normal	0	0%	1	3.3%	0.3 NS

NS: non-significant

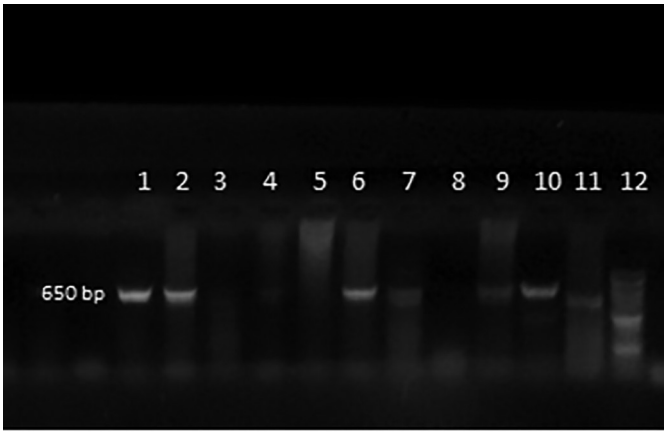

Fig. 1 – Results of the *rdxA* gene-PCR. Lanes 1, 2, 6, 7, 9, 10 and 11 contain rdxA gene-positive amplification products of 650 bp; lanes 3, 4, 5 and 8 are *rdxA*-negative; lane 12 is the 100 bp molecular weight marker.

pylori isolates. The difference of results may be explained by several factors. Firstly, different age groups were included in the two studies. In our study, we included patients > 18 years old[12]. In the study of Sherif *et al.* patients were 2-17 years old and were referred to the Abu-Resh Children's Hospital[12]. This age group is more frequently exposed to metronidazole for the treatment of parasitic infections and sometimes are highly exposed to this drug during frequent treatment schedules[13]. The second factor is the study design. In the study of Sherif *et al*. the gastric biopsies were incubated and the *in vitro* susceptibility was performed. In our study, metronidazole resistance was detected through a *rdxA* gene mutation. The finding of resistant strains to metronidazole by the *in vitro* methods is not clinically reliable as *in vitro* susceptibility tests have a tendency to overestimate resistance. For this reason and also due to the lack of a clinical-bacteriological correlation, the Maastricht IV Consensus Report has discouraged the routine metronidazole susceptibility testing[1]. The existence of other mechanisms associated with the *rdxA* gene inactivation such as the insertion of a transposable element called Mini-IS605[3] is also noteworthy.

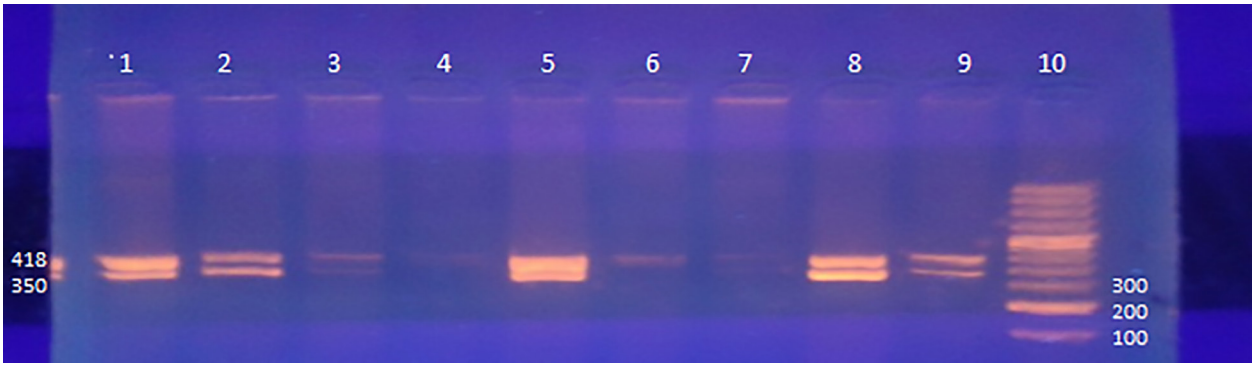

Fig. 2 - Detection of mutations associated with clarithromycin resistance in *Helicobacter pylori* strains by PCR-RFLP. Electrophoresis in a 3% agarose gel of 23S rDNA-PCR-RFLP. Lane 10 is the 100-bp molecular weight marker. Lane 9 is the positive control. **Lanes** 1, 2, 3, 5, 7 and 8 show the amplifications products of samples containing the A2142G mutation after digestion with the *MboII* restriction enzyme. Lanes 4 and 6 are the 23S-PCR negative controls.

Regarding clarithromycin resistance 55.7% of *H. pylori* positive cases harbored a 2142G mutation known to confer clarithromycin resistance with a rise in the resistance percentage from 4% in Sherif *et al.* study.[12] Macrolides are widely used to treat upper respiratory tract infections in Egypt.

In our study the A2142G mutation was the commonest among the tested samples while the A2143G mutation was not found among the isolates, contrasting with other authors who reported that the commonest mutations were A2143G and A2142G found in equal percentages among the tested isolates[14]. Acosta *et al*. 2014 reported that the A2143G mutation was more frequent than the other mutation A2142G, and both were the most frequent mutations, while Hansomburana *et al*. (2012) documented that the A2142G mutation was more frequent than the A2143G one[15,16].

Although clarithromycin resistance was found in 55.7% of *H. pylori*-positive cases, this percentage could be still underestimated due to the presence of other mutations which were not detected in our study. The eradication of *H. pylori* strains presenting high resistance rates to macrolides, or metronidazole could be reached by the use of approved tetracycline- or amoxicillin-containing regimens replacing the standard triple therapy[17].

In conclusion, the discovery of this high prevalence of genetic mutations that may confer resistance to metronidazole and clarithromycin, is most likely due to the misuse of macrolides and metronidazole. The clinicians' awareness of the isolates current antimicrobial susceptibility profiles will improve the treatment strategies and the patients' outcomes.

DISCLOSURE STATEMENT

No competing financial or conflict of interests exist.

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