British Microbiology Research Journal
15(2): 1-9, 2016, Article no.BMRJ.26157
ISSN: 2231-0886, NLM ID: 101608140

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Detection of A2142G and A2143G Substitutions among Clarithromycin-resistant *Helicobacter pylori* Strains Isolated from Egyptian Patients

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Authors' contributions

This work was carried out in collaboration between all authors. All authors planned and designed the study, wrote the protocol, collected the samples, performed the practical laboratory activities, participated in the interpretation of the results and analysis, drafted and critically revised the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2016/26157
Editor(s):
(1) Ng Zhi Xiang, Department of Biomedical Sciences, Faculty of Medicine, MAHSA University, Malaysia.
Reviewers:
(1) Zhijun Bao, Gastroenterology, Fudan University, China.
(2) Bruna Maria Roesler, State University of Campinas, Campinas, SP, Brazil.
(3) Virginia Montero- Campos, Instituto Tecnológico de Costa Rica, Cartago, Costa Rica.
(4) Nagahito Saito, Hokkaido University, Sapporo, Japan.
Complete Peer review History: http://sciencedomain.org/review-history/14880

Original Research Article

Received 3rd April 2016
Accepted 26th May 2016
Published 2nd June 2016

ABSTRACT

Background: Clarithromycin is the most commonly recommended antibiotic in *Helicobacter pylori* (*H. pylori*) eradication regimens, but the prevalence of clarithromycin-resistant *H. pylori* is increasing. Clarithromycin-resistance is associated with mutations in the 23S rRNA gene. The study aimed to examine gene mutations (A2142G and A2143G) of *H. pylori* 23S rRNA responsible for resistance to clarithromycin.
Materials and Methods: The study was carried out by collecting 53 *H. pylori* isolates. Isolation, identification and antimicrobial susceptibility to clarithromycin were done by standardized methods. Resistant strains were analysed for mutations in the 23S rRNA gene by polymerase chain reaction-based restriction fragment length polymorphism and sequencing.
Results: *H. pylori* isolates were recovered from 91.4% of studied patients. About 64% were clarithromycin-resistant strains. The minimum inhibitory concentration (MIC) values of all clarithromycin-resistant isolates ranged from 1.5 to 8µg/ml. Primary clarithromycin-resistant isolates

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only showed a single type of point mutation (A2143G). In contrast, secondary isolates had dual diversity of 23S rRNA gene mutation types (A2142G and A2143G).
Conclusion: Secondary clarithromycin-resistant isolates show a greater variety of 23S rRNA gene mutation types than primary isolates.

Keywords: Helicobacter pylori; 23S rRNA; clarithromycin-resistance; gene mutations; sequencing.

1. INTRODUCTION

Infection with *Helicobacter pylori* (*H. pylori*) is the major cause of chronic gastritis and peptic ulceration and an important factor in the development of gastric cancer [1]. Infection with *H. pylori* can be effectively treated by the combination of a proton pump inhibitor with multiple antibiotics. The first-line regimen consists mainly of a triple therapy, and clarithromycin is one of the most widely used components [2]. Therefore, study of resistance to clarithromycin is important [3,4]. Clarithromycin is recognized as the key antibiotic for *H. pylori* treatment, because of its powerful bactericidal effect *in vitro* compared with the other available molecules [4]. Clarithromycin acts by binding to the peptidyl transferase region of 23S rRNA and inhibits bacterial protein synthesis [5].

Unfortunately, primary clarithromycin resistance, no history of previous clarithromycin administration, is increasing worldwide, and it has been regarded as the main factor reducing the efficacy of eradication therapy [5]. Secondary resistance was defined as the resistance in patients who received at least one eradication therapy [3].

The predominant reason for eradication failure in patients treated with monotherapy clarithromycin is the development of resistance during treatment due to mutations in the 23S rRNA**.** Most prevalent among mutations are at A2142G and A2143G in the 23S rRNA gene [2]. However, other point mutations outer of domain V were reported with *H. pylori* resistant to clarithromycin, such as G2224A, C2245T, T2289C, and T2182C [6].

The prevalence of mutant strains among the clarithromycin-resistant *H. pylori* varies in different parts of the world. The prevalence of the A2143G mutation has been reported as 44% to 67% in Brazil [7]. However studies in Japan showed that, more than 80% of the mutant strains are in the position A2143G [8]. Spanish patients have one of the highest levels of clarithromycin-resistance reported in Europe. In Spain, similar to other European countries, including France, Portugal, Poland, Turkey and Bulgaria, increase of resistance to clarithromycin has been observed. In Northern European countries, this increase was not observed [9]. This difference probably depends on macrolide consumption.

The aim of this study was to assess mutations within domain V of the 23S rRNA gene (A2142G and A2143G) in clarithromycin-resistant clinical *H. pylori* isolates from Egyptian patients by analysing PCR products obtained from the clarithromycin-resistant isolates by PCR-RFLP and confirming the results by nucleotide sequencing, and to characterize the influence of each type of mutation on the MIC among primary and secondary clarithromycin-resistant *H. pylori*.

2. MATERIALS AND METHODS

2.1 Patients

A total of 58 adult patients presenting upper gastrointestinal symptoms were enrolled in the study. Fifteen patients had no history of previous triple therapy. Forty-three patients were subjected to previous eradication therapy, which included clarithromycin. The inclusion criterion was the indication of endoscopy for the examination of dyspeptic symptoms. The exclusion criteria were as follows: age under 18; previous gastric surgery; previous *H. pylori* eradication treatment for primary clarithromycin-resistant group; consumption of antibiotics in the previous month; or consumption of antisecretory drugs, bismuth salts, or sucralfate in the previous 2 weeks. A history of bleeding or a coagulation disorder that contraindicated biopsy sampling was also a reason for exclusion.

Permission and signature from all patients included in the study and ethical approval from the Local Ethical Committee of Menoufia University Hospital, Shebin El-Kom Teaching Hospital Hospital and 6th October University Hospital were obtained for the use of the specimens. All patients enrolled in the study

underwent upper gastrointestinal endoscopy between May 2014 and August 2015 at the Gastroenterology units in Menoufia University Hospital, Shebin El-Kom Teaching Hospital Hospital and 6th October University Hospital. The patients' serum samples were collected and tested for the presence of *H. pylori* IgG antibody by using quantitative enzyme immunoassay (EIA) (Monobind 1425-300 USA). Three biopsies, two from the antrum and one from the corpus, were obtained from each patient. One of the antrum specimens was used for the rapid urease test and the other two were used for bacterial culture and antibiotic susceptibility testing [10].

2.2 Isolation and Identification of *H. pylori* from Biopsies

The specimens taken from patients were transported immediately to the microbiology laboratory in 0.5 ml of glycerol-supplemented brain–heart infusion broth in individual microtubes. Biopsy materials were grounded by a sterile glass grinder. Cultures were performed on a commercialized Columbia Blood Agar Base (Oxoid) supplemented with 10% horse blood (bioMe´rieux) plus *H. pylori* selective supplement (Dent, SR0147E), under microaerophilic conditions at 37℃ in anaerobic jar flushed with a microaerophilic gas mixture (5% O_2, 10% CO_2, and 85% N_2) for a maximum of 6 days. *H. pylori* colonies were identified by standard methods [11]. *H. pylori* isolates were defined as Gram-negative, spiral-shaped bacilli that were catalase and oxidase positive and rapidly (less than 1 h) urease positive [12]. All positive cultures were included in the following steps.

2.3 Determination of Clarithromycin-resistant *H. pylori* Strains

MICs of clarithromycin (Abbott Laboratories, Abbott Park, Ill.) were determined by using agar dilution method [13]. Briefly, the bacteria were subcultured on Mueller–Hinton agar plates (Oxoid) supplemented with 10% horse blood (bioMe´rieux) for 48 h. The bacterial suspension, adjusted to 1×10^8 CFU, was inoculated directly onto each antibiotic-containing agar dilution plate according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [14]. After 72 h of incubation at 37℃ under microaerophilic conditions, MIC was determined. The resistance breakpoint for clarithromycin was set at MIC $\geqslant$ 1.0 µg/ml, intermediate if MIC = 0.5 µg/ml and sensitive at MIC $\leqslant$ 0.25 µg/ml [14].

2.4 DNA Extraction and PCR Amplification

DNA was extracted from pure positive *H. pylori* cultures. Colonies were suspended in 1 ml of sterile saline (NaCl 0.85% Medium, bioMérieux, France) and centrifuged (12000 rpm/3 min). Total bacterial genomic DNA was extracted by using Thermo Scientific Gene Jet genomic DNA purification Kit (Thermo K0721) according to the manufacturer's recommendations. The eluted DNA was stored at –20℃ until use. The conserved 1402 base pair (bp) region of the *H. pylori* 23S rRNA gene between nucleotide positions 1445 and 2846 (GenBank accession no. U27270) was amplified using forward primer 5′-AGTCGGGGACCTAAGGCGAG-3′ and reverse primer 5′-TTCCCGCTTAGATGCTTT CAG-3′ that were designed and synthesized by Qiagen (Germany) [15]. PCR was carried out in a total volume of 50µl consisted of 25 µl Maxima® Hot Start PCR Master Mix (2X) (Thermo K1051), 1 µl forward primer, 1 µl reverse primer, 5µl template DNA and 18 µl water nuclease-free. PCR conditions were: one initial denaturation/enzyme activation cycle at 95℃ for 10 minutes followed by 35 cycles at 95℃ for 30 seconds, 54℃ for 1 minute, and 72℃ for 90 seconds. A final elongation step was at 72℃ for 10 minutes, using Thermo Scientific Ark Tick thermal cycler (Finland). Amplified fragments were visualized on a 1% agarose gel electrophoresis stained with ethidium bromide. A DNA ladder (100 bp) was used to estimate allele sizes in base pairs (bp) for the gel. *H. pylori* ATCC®700392™ was used as positive control for identification of *H. pylori* and *H. pylori* ATCC®43504™ was use as negative control (LGC standards, United Kingdom).

2.5 RFLP Analysis

Analysis of the 23S rRNA gene mutations is based on the generation of restriction sites for two restriction enzymes: *Mbo*II and *Bsa*I, which correspond to the base substitutions characteristic of clarithromycin resistance from A to G at positions 2142 and 2143, respectively. PCR products were digested with *Mbo*II (5′...GAAGA $(N)_8^{\nabla}$...3′) and *Bsa*I (5′...GGTCTC $(N)_1^{\nabla}$...3′) restriction enzymes (BioLab, Beverly, MA, USA) to detect A2142G and A2143G point mutations, respectively. PCR products were analysed on 1% agarose gels. *Mbo*II-digested PCR products containing A2142G mutation are expected to yield two fragments (710 and 692

bp). *Bsa*I-digested PCR products containing A2143G mutation are expected to display three fragments (693, 395, and 314 bp) [16].

2.6 Sequence Analysis

PCR products were purified by using Thermo Scientific Gene JET™ PCR Purification Kit (Thermo K0701) and subjected to sequencing reactions using BigDye™ Terminator version 3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). The sequences were analysed using ABI 3730xl DNA sequencer machine (Applied Biosystems, HITACH) [16]. All analyses were performed in duplicate.

2.7 Statistical Analyses

Computer SPSS program version 17 was used. The results were expressed by applying Chi-square test and P values. $P < 0.05$ was considered to be significant.

3. RESULTS

The demographic and clinical data of the 58 patients with mean age of 35±14.9 years old enrolled in the study are presented in Table 1. The higher percentages were presented among female patients (56.9%), patients from rural area (65.5%), patients with low socioeconomic status (51.7%), and non-smoker patients (65.5%) with no significant difference ($P > 0.05$). About 57% of patients were associated with co-morbidities (Diabetes mellitus, hypertension, obesity, …etc). Only 8.6% of patients had previous history of GIT endoscopy while 34.5% had family history of *H. pylori* infection and 25.8% had family history of peptic ulcer disease with no significant difference ($P > 0.05$). Forty-three patients had previous history of clarithromycin treatment.

Testing of the patients' serum samples for the presence of *H. pylori* IgG antibody by using quantitative enzyme immunoassay (EIA) recovered *H. pylori* infection among 55 out of 58 (94.8%) studied patients. While culturing results yielded 53 *H. pylori* isolates (91.4%) out of 58 studied patients. Antimicrobial susceptibilities pattern of *H. pylori* isolates against clarithromycin regarding patients with prior clarithromycin treatment (no. = 38) showed that, 19 isolates (50%) were clarithromycin-resistant strains (MIC ≥ 1.0 µg/ml), 8 isolates (21.1%) showed intermediate clarithromycin-resistance (MIC = 0.5 µg/ml) and 11 isolates (28.9%) were sensitive (MIC < 0.5 µg/ml) to clarithromycin (Table 2). The fifteen patients with no history of previous triple therapy showed primary clarithromycin-resistant (100%). There was no significant difference ($P > 0.05$) regarding degree of susceptibility to clarithromycin in patients with prior clarithromycin treatment. While there was significant difference ($P < 0.05$) regarding degree of susceptibility to clarithromycin in total number of patients.

Table 1. Demographic and clinical characteristics of studied patients

Variables		No. (%)	P value
Age	35±14.9 years old		
Gender	Male	25 (43.1)	
	Female	33 (56.9)	
Residence	Urban	20 (34.5)	
	Rural	38 (65.5)	
Socioeconomic status	High or moderate level	28 (48.3)	
	Low level	30 (51.7)	
Smoking	+ve	20 (34.5)	> 0.05
	-ve	38 (65.5)	
Family history of *H. pylori* infection	+ve	20 (34.5)	
	-ve	38 (65.5)	
Family history of peptic ulcer disease	+ve	15 (25.8)	
	-ve	43 (74.2)	
Previous history of clarithromycin treatment	+ve	43 (74.2)	
	-ve	15 (25.8)	
Previous history of GIT endoscopy	+ve	5 (8.6)	
	-ve	53 (91.4)	
Associated co-morbidities	+ve	33 (56.9)	
	-ve	25 (43.1)	

Table 2. Antimicrobial susceptibilities pattern of *H. pylori* isolates against clarithromycin

Patients	Susceptible MIC < 0.5 µg/ml		Intermediate MIC = 0.5 µg/ml		Resistant MIC ≥ 1.0 µg/ml		P value
	No	%	No	%	No	%	
Prior clarithromycin treatment (no. = 38)	11	28.9	8	21.1	19	50	> 0.05
No prior clarithromycin treatment (no. = 15)	0	-	0	-	15	100	-
Total (no. = 53)	11	20.7	8	15.1	34	64.2	< 0.05

Two gene mutations, A2142G and A2143G, were observed among 32 out of 53 isolated *H. pylori* (60.4%). All 32 strains contain A2142G and/or A2143G mutations were clarithromycin-resistant strains (94.1%). A2142G mutation was observed among 5 strains (15.6%) alone, A2143G was observed among 7 strains (21.9%) alone and dual mutation was observed among 20 strains (62.5%) of clarithromycin-resistant *H. pylori* (Table 3). No mutation was observed among strains showed intermediate resistance or sensitivity to clarithromycin.

The distribution of mutations was 7/15 (46.7%) of primary clarithromycin-resistant isolates (5 strains had A2142G and 2 strains had A2143G) and 25/38 (65.8%) of secondary clarithromycin-resistant isolates (5 strains had A2143G and 20 strains had dual mutations). Primary clarithromycin-resistant isolates only showed a single type of point mutation. Most of secondary clarithromycin-resistant isolates showed dual mutation (80%) (Fig. 1).

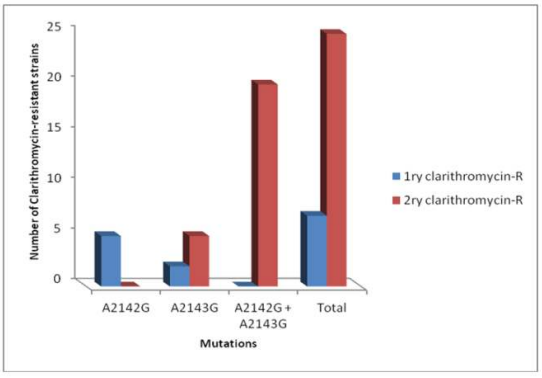

Fig. 1. Distribution of mutations among primary and secondary clarithromycin-resistant isolates

The MIC values of all the clarithromycin-resistant isolates ranged from 1.5 to 8 µg/ml. The strains with A2143G mutation had lower average MIC values than strains with A2142G mutation (2.9 µg/ml and 5.1 µg/ml, respectively). The strains with dual mutations had MIC average of 6.5 µg/ml (Fig. 2).

Table 3. Prevalence of 23S rRNA mutations among clarithromycin-resistant *H. pylori* strains

Mutation		Clarithromycin-resistant *H. pylori* strains	
		Number	%
Single point mutations	A2142G	5/32	15.6
	A2143G	7/32	21.9
Dual mutation	A2142G + A2143G	20/32	62.5
Total mutation		32/34	94.1
Undetermined mutation		2/34	8.9

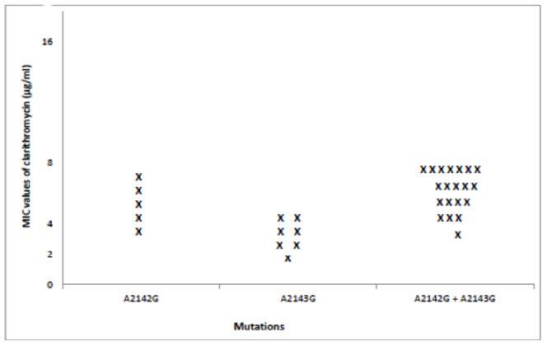

Fig. 2. Relation between phenotypic and genotypic resistance patterns of studied *H. pylori* isolates

Sequence analysis of restriction sites of the amplified V domain of the 23S rRNA gene of the resistant strains showed restriction sites that could be digested with *Mbo*II and *Bsa*I. Digestion of the amplified PCR product with *Mbo*II produced two fragments of 710 and 692 bp sizes in strains with the A2142G mutation. No fragment was produced with the A2143G (Fig. 3). Resistant isolates with the A2143G mutation produced three fragments of 693, 395 and 314

bp when digested with *Bsa*I. No restriction fragment was produced in strains with A2142G mutations (Fig. 4). None of the clarithromycin-sensitive isolates produced restriction fragments when digested with *MboII* and *BsaI*, which indicated the absence of A2142G and A2143G mutations.

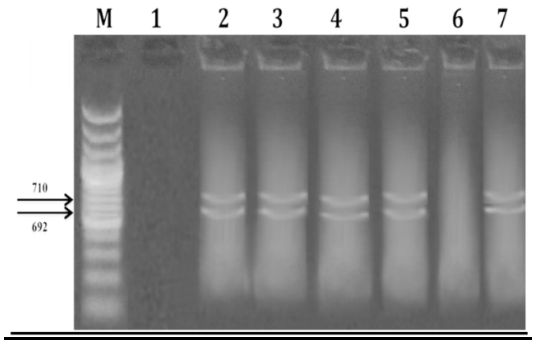

Fig. 3. Detection of the 23S rDNA A2142G substitutions by *Mbo*II– mediated polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP)
M line: 100 bp DNA ladder. Lane 1: no template. Lanes 2, 3, 4, 5 and 7 have A2142G mutant (MboII digested)

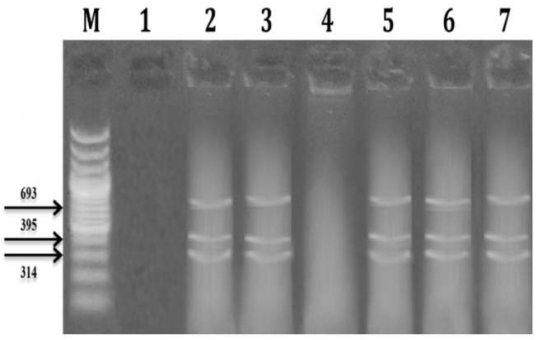

Fig. 4. Detection of the 23S rDNA A2143G substitutions by *Bsa*I-mediated polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP)
*M line: 100 bp DNA ladder. Lane 1: no template. Lanes 2, 3, 5, 6 and 7 have A2143G mutant (Bsa*I *digested)*

4. DISCUSSION

The development of clarithromycin resistance is a major cause of *H. pylori* treatment failure [4,17]. The resistance pattern of *H. pylori* varies according to the geographical regions [13]. *H. pylori* resistance to clarithromycin is predominantly related to the point mutations in the peptidyl transferase encoding region of V domain of 23S rRNA gene. The most prevalent point mutations responsible for this process are A2143G, A2142G, and A2142C [18,19]. Moreover, other mutations such as A2115G, G2141A, C2147G, T2190C, C2195T, A2223G, and C2694A have been identified among *H. pylori* strains resistant to clarithromycin, although their role in the mechanism of resistance remains unclear [8,20].

The present study aimed at determining the genetic variability of domain V of the 23S rRNA gene (A2142G and A2143G) in clarithromycin-resistant clinical *H. pylori* isolates from Egyptian patients by analyzing PCR products obtained from the clarithromycin-resistant isolates by PCR-RFLP and confirming the results by nucleotide sequencing, and to characterize the influence of each type of mutation on the MIC. The discovery of genomic variability in *H. pylori* offers simple, accurate and efficient molecular typing techniques that are useful in both epidemiological and clinical studies [12,21].

Our study revealed that *H. pylori* isolates were recovered from 91.4% of studied patients. Females and patients with low socioeconomic status are associated with high incidence of *H. pylori* infection. Also, 65.5% of patients infected with *H. pylori* have family history of *H. pylori* infection. About 64% of our isolated strains were clarithromycin-resistant strains. Ghaith and his colleagues [22] revealed that, the prevalence of *H. pylori* infection among 100 patients was 70%; clarithromycin-resistance was detected in 39/70 (57.7%) of positive *H. pylori* isolates. While *Zaki* and his colleagues [4] reported that the isolated *H. pylori* had resistance for clarithromycin among 46.2%.

Our study showed that, the fifteen patients with no history of previous triple therapy had primary clarithromycin-resistant (100%). Similar result was reported by Tüzün et al. [23] in his geographical area (southeastern Anatolia). While in Tunisia, low rate of primary clarithromycin-resistance was reported (15.4%) [24]. Also in contrast to our results, by analysis the results of *Agudo and his colleagues* [25], *H. pylori* isolates from previously treated patients were more resistant to clarithromycin than *H. pylori* strains isolated from patients who had not received any treatment (58.3% and 21.5%, respectively).

No gene mutations were observed among strains showed intermediate resistance or sensitivity to

clarithromycin. A2142G mutation was observed among 15.6% alone, A2143G was observed among 21.9% alone and dual mutation was observed among 62.5% of clarithromycin-resistant *H. pylori*. Similar results were reported by Zaki et al. [4] who found that, the most common types of mutation were A2143G (53.4%) followed by A2142G (35.7%). Also, Agudo et al. and Raymond et al. [8,26] concluded that A2143G mutation was more common than A2142G one. On the other hand, Klesiewicz et al. [27] reported that, the frequencies of A2143G and A2142G mutations were the same in all isolates tested. The discrepancies between the results of authors may have arisen from either geographical origin of the isolates or limited number of tested strains in some studies.

Primary clarithromycin-resistant isolates only showed a single type of point mutation (71.4% had A2142G and 28.6% had A2143G). In contrast, 80% of secondary isolates had dual diversity of 23S rRNA gene mutation types, A2143G and A2142G and only 20% had a single type of point mutation, A2143G. Dual mutations in secondary isolates may be a result of exposure to clarithromycin for long periods. It is interesting that all clarithromycin-resistant secondary isolates harboring dual mutations have A2143G mutation. Based on this finding, it is possible that A2143G plays a major role of clarithromycin resistance but other mutations may affect accessory roles. Therefore, our research confirms the reports of several authors that the predominant mutations responsible for clarithromycin resistance in *H. pylori* are A2143G and A2142G [13].

Our phenotypic analysis revealed the occurrence of two clarithromycin-resistant strains (8.9%) without any of the assayed mutations. In these isolates, resistance to clarithromycin might be associated with other less common mutations or with the efflux mechanism. Therefore, further study is required.

In this study, the MIC values of all the clarithromycin-resistant isolates ranged from 1.5 to 8 µg/ml. It showed that the strains with A2143G mutation had lower average MIC values than strains with A2142G mutation (2.9 µg/ml and 5.1 µg/ml, respectively). The strains with dual mutations had MIC average of 6.5 µg/ml. Results of Klesiewicz et al. [27] study showed that, the strains with A2143G mutation had lower average MIC values than strains with A2142G mutation (6 µg/ml and 30 µg/ml, respectively). These results are also consistent with those reported by other researchers who concluded that A2143G point mutation was correlated with lower clarithromycin MIC values than A2142G one [20,28,29,30].

Our study has a limitation that the relatively low number of patients and a possible selection bias by investigating patients from an academic institution. That is, there could be a possibility of overestimation of prevalence because patients in academic institution usually have multiple underlying diseases, in which patients took more antibiotics. The increased primary and secondary antibiotic resistance of *H. pylori* is going on in Egypt, and it will become a significant limitation for effective eradication of *H. pylori* in future.

5. CONCLUSION

In conclusion, A2142G and A2143G were prevalence point mutations in the 23S rRNA gene of *H. pylori* isolates associated with clarithromycin-resistance in Egyptian patients. However, primary clarithromycin-resistant isolates only showed single type of mutation. In contrast, dual mutations occur in secondary isolates indicating that secondary isolates have a greater diversity of 23S rRNA gene mutation types than primary isolates. The high prevalence of clarithromycin-resistance may mandate changing of the standard clarithromycin-containing triple therapy.

ACKNOWLEDGEMENT

Thanks to the staff at the Gastroenterology units in included Egyptian Hospitals, especially Dr. Moamena S. El-hamouly, Faculty of Medicine, Menoufia University.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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