Egyptian Journal of Medical Microbiology Volume 27 / No.3 / July 2018 13-20 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Prediction of *Helicobacter Pylori* Clarithromycin Resistance by Detection of Point Mutations in *23S rRNA* gene

[1]Mostafa A. Soltan, [1]Marwa A. Mansour, [2]Tarek I. Zahir, [3]Hayam E. Rashed, [1]Yasmin A. Fahmy

[1]Medical Microbiology and Immunology Department; [2]Tropical Medicine Department; [3]Pathology Department, Faculty of Medicine, Zagazig University

Key words:
Helicobacter pylori; 23S rRNA; clarithromycin-resistance; point mutations

Corresponding Author:
Yasmin Ahmed Fahmy
Medical Microbiology and Immunology Department
Tel.: 01113320914
yasminfahmy@yahoo.com

ABSTRACT

Background: *Clarithromycin is one of the most common drugs recommended as first-line eradication therapy for H. pylori infection but the prevalence of clarithromycin resistant H. pylori is increasing. Clarithromycin-resistance is associated with point mutations in the 23S rRNA gene.* ***Objective:*** *Detecting the frequency of H. pylori clarithromycin resistance and demonstrating clarithromycin resistant genotypes A2142G and A2143G.* ***Methodology****: This study included 123 patients during upper gastroduodenoscopy in the Endoscopy Units of Zagazig University Hospitals. Three gastric biopsies were obtained from each patient. H. pylori infection was detected by rapid urease test, histopathology, and PCR. The biopsy was considered H. pylori positive if both rapid urease test and histopathology were positive. Clarithromycin resistance was assessed by Polymerase chain reaction-amplification and restriction fragment length polymorphism (PCR-RFLP).* ***Results****: Out of 123 patients, 71 patient were infected with H. pylori. Clarithromycin resistance was detected in 47 of 71 positive H. pylori strains (66.19 %), the A2142G point mutation was detected in 37 cases (52.11%) , A2143G in 10 cases (14.08 %) while dual point mutations were observed in 2 cases (2.81%).* ***In conclusion:*** *High prevalence of clarithromycin resistant H. pylori among Egyptian patients .The A2142G is the most frequent detected point mutations involved in clarithromycin resistance in our country.*

INTRODUCTION

H. pylori is responsible for different gastric diseases, such as chronic gastritis, gastric and duodenal peptic ulcer, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (MALT) [1]. The World Health Organization (WHO) considered this bacterium as carcinogenic (group 1) and reported that *H. Pylori* are responsible for 75 % of cases of gastric cancer[2].

Clarithromycin is recommended as first-line eradication therapy for *H. pylori* infection, and has been often used in combination with a proton pump inhibitor and amoxicillin or metro-nidazole [3]. Clarithromycin inhibits the protein synthesis by binding to the 50S bacterial ribosomal subunit. Its resistance is involved in failure of *H. pylori* therapy[4]. Resistance to clarithromycin may develop when substitutions in one nucleic acid at or near this binding site on the ribosome prevent the drug from binding, thereby making it ineffective [5].

Point mutations in domain V of the *23S rRNA* gene are responsible for the resistance of *H. pylori* to clarithromycin, with the three major point mutations being A2142G, A2143G, and A2141G.The most frequent of these are transitions of adenine to guanine at the 2143 or 2142 positions, which account for > 80% of clarithromycin resistance seen worldwide and about 90 % of resistance seen in developed countries [6]. Many molecular-based methods are now available to assess clarithromycin resistance in *H. pylori*, such as polymerase chain reaction-Restriction fragment length polymorhism (PCR-RFLP), real-time PCR, DNA sequencing[7]. The PCR-based molecular techniques is quicker than microbiological susceptibility testing, and they can be performed directly on gastric biopsies and gastric juice [5].

This study aims to predict *Helicobacter Pylori* clarithromycin Resistance by detection of point mutations in *23S rRNA* gene.

METHODOLOGY

Study setting

This cross-sectional study was conducted at Gastrointestinal Endoscopy Units at Zagazig University Hospitals, Molecular Biology Laboratory at Medical Microbiology and Immunology Department, Histopathology Laboratory at Pathology Department

and Scientific and Medical Research Center , Faculty of Medicine, Zagazig University in the period from January 2016 to September 2017.

Ethical consideration

Approval for performing the study was obtained from Tropical Medicine Department and General Medicine Department, Zagazig University after taking Institutional Review Board (IRB) approval. A written informed consent was obtained from all patients.

Patients:

The study included patients who were doing upper gastro-duodenoscopy for upper abdominal pain or dyspeptic symptoms and patients who did not take any antibiotic for *H. pylori* infection in the past 2 weeks. The study excluded the patients with severe concomitant diseases such as chronic renal failure, decompensated heart failure and previous gastric surgery or patients who were under treatment with antisecretory or proton pump inhibitors during the last month prior to the study. Also pregnant or lactating women were excluded.

Complete medical history was taken from the patients. Gastric biopsies were obtained from 123 patients during upper gastro-duodenoscopy including 55 males and 68 females with their ages range from 19 to 70 years.

Collection of samples:

From each patient, three gastric biopsy specimens were taken from the antrum of the stomach using a disinfected endoscope (GIF XQ230; Olympus, Center Valley, PA). The first biopsy was examined by rapid urease test (RUT) and the second one was placed in clean container containing 10 % formalin for histopathological examination for detection of *H. pylori*. The third biopsy was placed in 0.1 mL of sterile saline solution, transported and frozen at -80 C for further DNA extraction[8] at the Scientific and Medical Research Center.

Identification of *Helicobacter pylori:* This was done by the following methods:

Rapid Urease Test:

RUT was done by using commercial paper RUT according to the manufacturer's protocol **(Helicotec UT® Plus; Catalog No. HUP01, strong Biotech Corporation, Taiwan)**. The biopsy specimen was transfered onto the test paper with the applicator included in the test kit. Color changes were observed within one hour. When the outer ring of the test paper changed color to pink or red, the test was considered positive, while when remained yellow in color, the test was considered negative[9].

Histopathological examination:

The second biopsy specimen was fixed in 10% formalin and sent to Histopathology Laboratory at Pathology Department. Paraffin embedded and multiple 4 mm-thick histological sections were obtained from each biopsy. Preparations were stained by Eosin and Haematoxylin stains for assessment of histopathologic alterations (figure 1) and Giemsa stain for *H. pylori* evaluation[10] (figure 2).

PCR:

DNA was extracted from biopsies using the Genomic DNA purification system according to the manufacturer's instructions **(QIAamp® DNA Mini kit; catalog No 51304, QIAGEN, Germany)** and stored at -20˚C until analysis. A sequence of 294 bp in the *ureC* gene was amplified using Maxime PCR PreMix Beads **(iNtron, Certified Company, Germany)**. Primer pair used for *ureC* amplification had the nucleotide sequence as follows:

forward primer, (5´AAGCTTTTAGGGGTGT T AGGGGTTT-3´) and reverse primer (5´- AA GCTTA CTTTCTAA CA CTAACGC-3´). The following were added to each tube containing a PCR bead: 6 µl of DNA template (50- 100 ng), 2µl equivalent of 100 pM of the two forward and reverse primers *(UreC-F, UreC-R)* (total 4 µl PCR primers) and pyrogen free water to a final volume of 20 µL. They were mixed well by automatic pipette followed by brief centrifugation to collect the content at the bottom of the tube. The amplification was carried out in a DNA thermal cycler **(Biometra, Germany).** The PCR conditions consisted of 1 cycle of 5 min at 93°C, followed by 35 cycles of 1 min at 93°C, 30 s at 55°C, 30 s at 72°C, and a final cycle of 10 min at 72°C. Amplified products were visualized on 2% agarose gel under UV light[11].

Detection of A2142G and A2143G point mutations by PCR-RFLP:

To detect clarithromycin resistance, 1400 bp fragment from an internal region of the *23S rRNA* gene was amplified followed by digestion with BsaI & MboII. The 1400 bp fragment normally has one restriction site for BsaI enzyme. If the gene is wild type, the enzyme produces a 1000 bp and a 400 bp fragments. If the A2143G point mutation occurs in 1400 bp fragment, the enzyme find two restriction sites and produces three fragments: a 700 bp, a 400 bp, and a 300 bp one. The 1400 bp fragment normally has no restriction site for MboII enzyme, therefore, if the gene is wild type, the 1400 bp remains undigested. But, if the A2142G point mutation exist, the enzyme find one restriction site in the 1400 bp fragment and digest it to two 700 bp fragments that look as one overlapping band in electrophorsis gel[12].

PCR for amplification of the 1400 bp fragment of 23S rRNA gene:

The 1400 bp fragment was amplified using Maxime PCR PreMix Beads **(iNtron, Certified Company, Germany)**. Primer pair used for the amplification had the nucleotide sequence as follows:

Cla-18 (AGTCGGGACCTAAGGCGAG) and Cla21 (TTCCCGCTTAGATGCTTTC AG). The following were added to each tube containing a PCR bead: 6 µl of DNA template (50- 100 ng), 2µl equivalent of 100 pM of the two forward and reverse primers (Cla 18, Cla 21)

(total 4 µl PCR primers) and pyrogen free water to a final volume of 20 µL and mixed well by automatic pipette followed by brief centrifugation to collect the content at the bottom of the tube. The amplification was carried out in a DNA thermal cycler **(Biometra, Germany).** PCR was performed as follow: Initial denaturation at 94°C for five min followed by 30 cycles of denaturation at 94°C for one min, annealing for one min at 58°C, extension at 72°C for one min. The final extension step was extended to five min at 72°C. Amplified products were visualized on 2% agarose gel under UV light.

RFLP protocol for detection of A2143G point mutation:

1 µg of the amplified 1400 bp fragment, 1 µl of BsaI-HF restriction enzyme ***(NEW ENGLAND BioLabs, UK)*** and 5 µl cut Smart Buffer (1X) were transferred to sterile epindorff with total reaction volume 50 µl. The tube then incubated at 37°C for 5-15 min. Then the reaction was stopped by adding 10 µl of 6X gel loading dye to 50 µl reaction. The restriction products were visualized on 2% agarose gel under UV light.

RFLP protocol for detection of A2142G point mutation:

1 µg of the amplified 1400 bp fragment , 1 µl of MboII restriction enzyme ***(NEW ENGLAND BioLabs, UK)*** and 5 µl cut Smart Buffer (1X) were transferred to sterile epindorff with total reaction volume 50 µl. The tube then incubated at 37°C for 5-15 min. Then the reaction was stopped by adding 10 µl of 6X gel loading dye to 50 µl reaction. The restriction products were visualized on 2% agarose gel under UV light.

Statistical Analysis:

Data were collected and coded, and all analyses were performed using Statistical Package for the Social Sciences software (SPSS version 20, Inc., Chicago, IL, USA). Data were entered as variables, represented by tables and graphs. Sensitivity, specificity, predictive values and accuracy of the diagnostic assays were calculated for all 123 patients in relation to the gold standard. Independent t-test was used for quantitative normally distributed data for detection difference between two different groups. Chi square and Fisher's exact was used to detect relation between different qualitative variable. Results were considered statistically significant when P (probability) values were equal to or less than 0.05 at confidence interval (CI) 95%.

RESULTS

Of the 123 patients undergoing upper gastrointestinal endoscopy, 68 were female, and 55 were male. Their ages were between 19 and 70 years, Mean ± SD (42.65± 13.5). Based on the gold standard used in this study,71(57.72%) patients were infected with *H. pylori*, while 52 (42.28%) were not infected.

The comparison of the three methods used for diagnosis of *H. pylori* infections by gold standard are shown in table (1). Demographic features, the clinical features and the endoscopic findings of *H. pylori* positive patients are shown in tables 2, 3 and 4 respectively.

The RFLP pattern showed that 37/71 (52.11%) of *H. Pylori* +ve patients has A2142G mutation (figure 3), 10/71 (14.08%) has A2143G mutation (figure 4) and 2/71 (2.81%) has dual mutation, so 47/71(66.19%) from *H Pylori* +ve patients were resistant to clarithromycin as shown in figure (5). We found no significant association between clarithromycin resistance and age (P>0.05) but there was significant association between clarithromycin resistance and sex with increase clarithromycin resistance among females (P= 0.03) as shown in table (5). Also we found no significant association between clarithromycin resistance and endoscopic finding except with duodenal ulcer present more in patients infected with clarithromycin resistant *H. pylori* (P= 0.012) as shown in table (6).

Table 1: Comparison of the three methods used for diagnosis of *H. pylori* infections by gold standard.

Methods		Gold standard (Rapid urease+ Histopathology)		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
		Positive	Negative					
Rapid urease test	Positive	71	6	100	88.4	93.42	100	95.12
	Negative	0	46					
Histology	Positive	71	26	100	50	73.19	100	78.86
	Negative	0	26					
PCR	Positive	50	0	70.42	100	100	71.23	82.92
	Negative	21	52					

Table 2: Comparison between *H. pylori* positive and negative patients as regard sex and age.

Variable	Rapid urease and Histopathology for *H. Pylori*				T	P value
	H. Pylori +ve (No =71)		*H. Pylori –ve* (No= 52)			
Age (years) Mean ± SD	**40.91± 13.02**		**45.01±13.92**		**1.67**	**0.09 NS**
Variable	**No**	**%**	**No**	**%**	χ^2	**P**
Sex:						
Female	**33**	**46.5**	**35**	**67.3**	**5.26**	**0.02***
Male	**38**	**53.5**	**17**	**32.7**		

No= number, NS: non significant, P of fisher's exact test, *Statistical significant difference

Table 3: Comparison between *H. pylori* positive and negative patients as regard clinical symptoms.

	H. Pylori + ve (No= 71)		*H. Pylori -ve* (No=52)		χ^2	P value
	No	*%*	No	*%*		
Epigastric pain	**62**	**87.3**	**39**	**75.0**	**3.1**	**0.07 NS**
Heart burn	**21**	**29.6**	**11**	**21.2**	**1.1**	**0.29 NS**
Hematemisis	**7**	**13.5**	**7**	**9.9**	**0.38**	**0.53 NS**
Dyspepsia	**10**	**14.1**	**14**	**26.9**	**3.15**	**0.07 NS**
Vomiting	**6**	**11.5**	**6**	**8.5**	**0.3**	**0.56 NS**

No= number, NS: non significant, P of fisher's exact test

Table 4: Comparison between *H. pylori* positive and negative patients as regard endoscopic findings.

	H. Pylori +ve (No= 71)		*H. Pylori –ve* (No=52)		χ^2	P value
	No	*%*	**No**	*%*		
Atrophic gastritis	**28**	**39.4**	**24**	**46.2**	**0.55**	**0.45 NS**
Gastric ulcer	**18**	**25.4**	**7**	**13.5**	**2.62**	**0.10 NS**
Duodenal ulcer	**16**	**22.5**	**6**	**11.5**	**2.47**	**0.11 NS**
Gastric mass	**2**	**2.8**	**2**	**3.8**	**0.1**	**0.75 NS**
Gastric polyp	**2**	**2.8**	**6**	**11.5**	**3.75**	**0.053 NS**

No= number, NS: non significant, P of fisher's exact test

Table 5: Comparison between patients with clarithromycin resistant and sensitive *H. pylori* isolates as regard age and sex.

	Patients with clarithromycin resistant *H. pylori* (No=47)		Patients with clarithromycin sensitive *H. pylori* (No=24)		T test	P value
Age : (year)						
Mean ± SD Rang	**40.83± 12.98 (20-60)**		**41.03± 13.33 (24 -60)**		**0.06**	**0.95**
Age group	**No**	**%**	**No**	**%**	χ^2	**P value**
< 45 (years)	**26**	**55.32**	**12**	**50.0**	**0.18**	**0.67**
≥ 45(years)	**21**	**44.68**	**12**	**50.0**		
Sex						
Male	**21**	**44.68**	**17**	**70.83**	**4.36**	**0.03***
Female	**26**	**55.32**	**7**	**29.17**		

**** Statistical significant difference***

Table 6: Comparison between patients infected with clarithromycin resistant and sensitive *H. pylori* isolates as regard endoscopic findings.

	Patients infected with clarithromycin resistant *H. pylori* (No=47)		Patients infected with clarithromycin sensitive *H. pylori* (No=24)		*Fisher s exact test*	*P value*
	No	%	No	%		
Atrophic gastritis	**18**	**38.29**	**13**	**54.17**	**0.94**	**0.33**
Gastric ulcer	**10**	**21.27**	**8**	**33.33**	**0.25**	**0.61**
Duodenal ulcer	**12**	**25.5**	**2**	**8.33**	**6.27**	**0.012***
Gastric mass	**0**	**0.0**	**2**	**8.3**	**3.16**	**0.07**
Gastric polyp	**2**	**4.25**	**0**	**0.0**	**1.34**	**0.24**

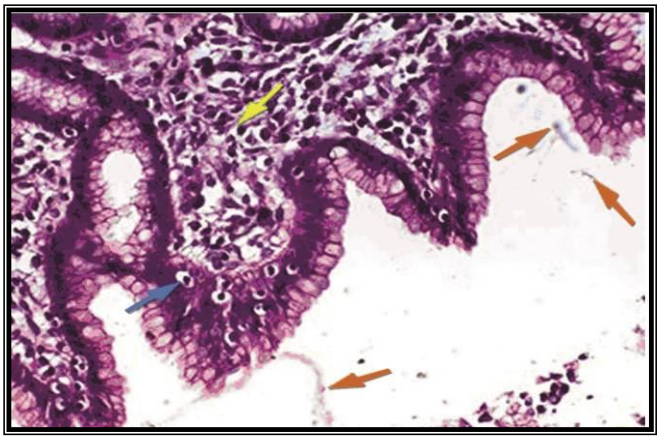

Fig. 1: Section of gastric mucosa revealed chronic gastritis associated with *H. pylori* (HE X 400).**Orange arrow:** *H. pylori*. **Blue arrow**: Intraepithlial lymphocyte. **Yellow arrow:** Inflamed lamina propria.

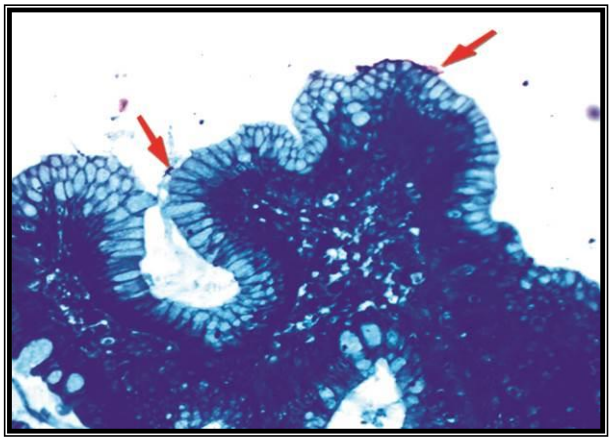

Fig. 2: Section of gastric mucosa (Giemsa X 400). **Red arrow:** *H. pylori* attached to gastric mucosa.

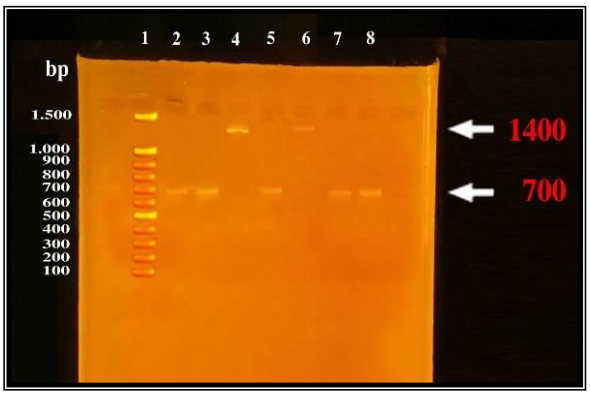

Fig. 3: PCR-RFLP patterns of 1400 bp fragments after digestion with Mbo II enzyme in order to detect A2142G point mutations in *23S rRNA* gene.

- Lane1:100 bp DNA ladder.
- Lane 2, 3, 5, 7, 8: one overlapping band (700 bp) positive for A2142G point mutation
- Lane 4 and 6: one band (1400 bp) negative for A2142G point mutation.

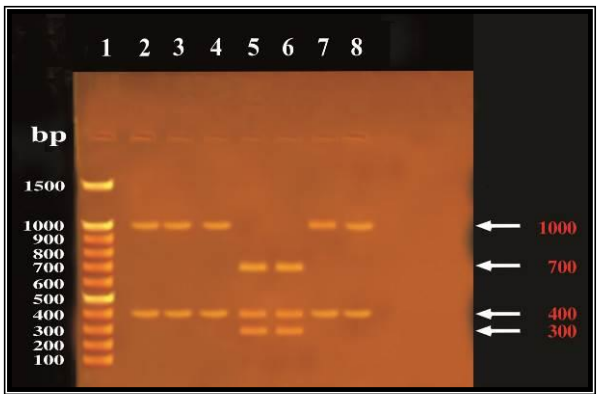

Fig. 4: PCR-RFLP patterns of 1400 bp fragments after digestion with Bsa I enzyme in order to detect A2143G point mutations in *23S rRNA* gene.

- Lane 1 :100 bp DNA ladder.
- Lane 5,6 : three bands (700 bp,400 bp,300 bp) positive forA2143G point mutation.
- Lane 2,3,4,7,8 :two bands(1000 bp,400bp) negative for A2143G point mutation.

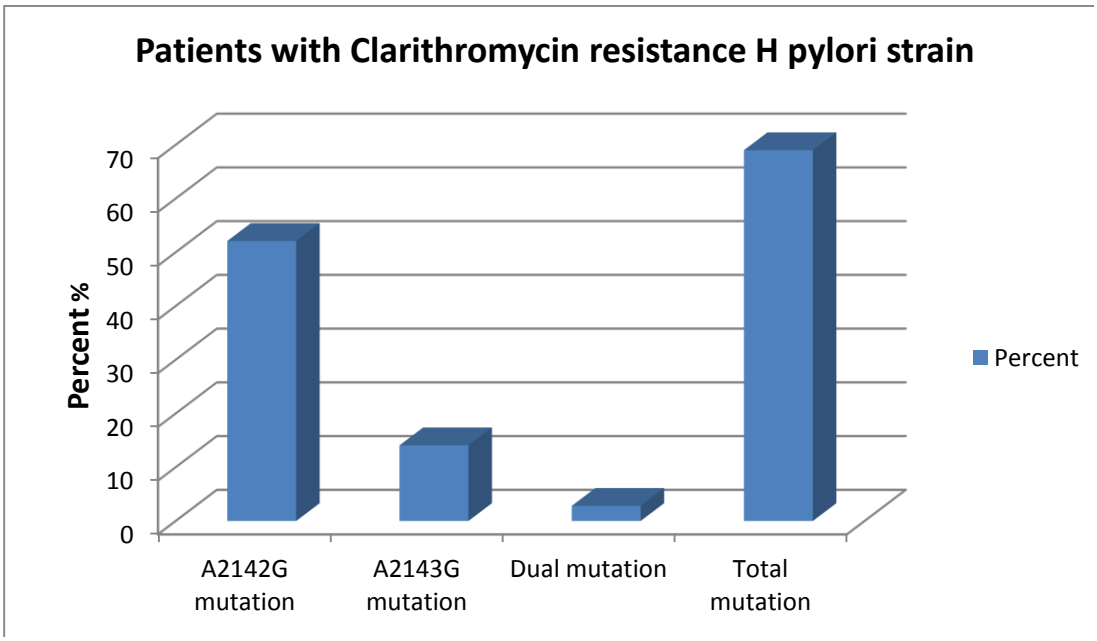

Fig. 5: Percent of *H. Pylori* +ve patients with clarithromycin resistance.

DISCUSSION

H. pylori infection is common in Egypt, and it can affect all age groups being more prevalent in adults than the pediatric population. The prevalence in asymptomatic population has been reported to reach up to 90% [13]. Although there are many methods for diagnosis of *H. pylori*, these methods differ in their cost, sensitivity and specificity [14]. So using of at least two methods with different principles for detecting *H. pylori* is recommended[15].

Considering the gold standard in the current study is combination of histopathological examination and rapid urease test, 71/123 (57.72%) patients were infected with *H. pylori.* This is in agreement with Ghaith and his colleagues[16] who revealed that the prevalence of *H. pylori* infection among 100 patients was 70%. Another study[17] also reported similar results where 91.4% of studied patients were infected with *H pylori*. Lower ratios were detected by Ramis and his colleagues [10] who reported that 48 (33.3%) patients were infected with *H. pylori.* The discrepancy in the percentages of positive *H. pylori* could be attributed to the difference in the number of clinical specimens investigated in different studies.

Regarding comparison of the three methods used for diagnosis of *H. pylori* infections by gold standard; rapid urease test and histopathology were the most sensitive methods for diagnosis of *H. pylori* where sensitivity of both tests were 100 % however PCR was the most specific test where specificity was 100 %. This is more or less in agreement with Khalifehgholi and his colleagues[18]. They reported that RUT was the most sensitive method followed by histopathology while RUT was the most specific test followed by PCR.

In our study, there was no statistically significant difference between patients with *H. pylori* positive as regard age (P = 0.09). This is in agreement with El-Masry and his colleagues[17] who showed that no significant difference as regard age. There was significant difference between patients with *H. pylori* positive as regard sex (P=0.02) , males (53.5%) more than female (46.5%). This is in agreement with Khashei and his colleagues [19] who found a statistical correlation (p < 0.014), male (50%) and female (50%). On the other hand, another study [20] found no significant difference as regard sex.

Concerning association between *H. pylori* positivity and clinical symptoms, there was no statistical significant difference between *H. pylori* positive and negative patients as regard symptoms, but there was increase in patients with epigastric pain and heart burn among patients who were *H. Pylori* +ve. This is in agreement with Ramzy and his colleagues [20] who found that no statistical significant relation between symptoms and *H. Pylori* positivity

Regarding association between *H. pylori* positivity and endoscopic findings, there no statistical significant difference between *H. pylori* positive and negative patients as regard endoscopic findings, there was increase in percent of atrophic gastritis, gastric ulcer and duodenal ulcer among *H. Pylori* +ve patients. Ramzy and his colleagues [20] agreed with us, they showed that no statistical significant difference in endoscopic findings in patients who were *H. Pylori* positive**.** On the other hand, another study [19] showed significant association

displaying that the frequency of *H. pylori* was highest (63%) in patients suffering from gastritis followed by 15% in gastric ulcer cases**.**

Clarithromycin is one of the most common drugs recommended as first-line eradication therapy for *H. pylori* infection but clarithromycin resistance is considered as the main factor involved in failure of *H. pylori* therapy. Point mutations in domain V of the *23S rRNA* gene are responsible for the resistance of *H. pylori* to clarithromycin, with the three major point mutations being A2142G, A2143G, and A2141G [4].

Regarding the prevalence of A2142G mutation and A2143G mutation among *H. pylori* positive strains in our study , 52.11% of *H. Pylori* +ve patients has A2142G mutation, 14.08% of patients has A2143G mutation and 2.81% of patient has dual mutation. So 66.19 % from *H. Pylori* +ve patients were resistant to clarithromycin. Ramzy and his colleagues [20] agreed with the current study, they found that 39 of 70 positive *H. pylori* samples (55.7%) samples had the A2142G mutation while none of the tested samples had the A2143G mutation. On the other hand**,** another study [21] found different results where the most common type of mutation was for A2143G (53.4%) followed by A2142G (35.7%). Diab and his colleagues [22] reported that 6.7% of positive *H. pylori* gastric biopsies had A2143G point mutation and none had A2142G point mutation. This work showed that clarithromycin resistance was higher among female patients with statistical significant difference. De Francesco and his colleagues [23] were supportive of our result; they found that clarithromycin resistance increased 3-fold in female. On contrary, Abadi and his colleagues[24] reported that no significant association with sex. The discrepancies between the results could be explained by the difference in the geographical origin of the isolates or limited number of tested strains in some studies.

CONCLUSION

Large percentages of Egyptian population are infected with *H. pylori*. High prevalence of clarithromycin resistant *H. pylori* among Egyptian patients. The A2142G is the most frequent detected point mutations involved in clarithromycin resistance in our country.

REFERENCES

1. Ayala G, Escobedo-Hinojosa WI, Cruz-Herrera CF, Romero I. Exploring alternative treatments for Helicobacter pylori infection. World J Gastroenterol. 2014; 20: 1450–69.
2. Garza-Gonza´lez E, Perez-Perez GI, Maldonado-Garza HJ, Bosques- Padilla FJ. A review of Helicobacter pylori diagnosis, treatment, and methods to detect eradication. World J Gastro-enterol. 2014; 20:1438–49.
3. Hirata K, Suzuki H, Nishizawa T, Tsugawa H, Muraoka H, Saito Y, et al. Contribution of efflux pumps to clarithromycin resistance in Helicobacter pylori. J. Gastroenterol. Hepatol . 2010; 25(1): S75–79.
4. De Francesco V, Zullo A, Hassan C, Giorgio F, Rosania R, Ierardi E. Mechanisms of Helicobacter pylori antibiotic resistance: An updated appraisal World J GastrointestPathophysiol15. 2011; 2(3): 35-41.
5. Yilmaz Ö, Demiray E. Clinical role and importance of fluorescence in situ hybridization method in diagnosis of H. pylori infection and determination of clarithromycin resistance in H. pylori eradication therapy. World J Gastroenterol. 2007; 13: 671–75.
6. Megraud F: H. pylori antibiotic resistance :prevalence,importance ,and advances in testing. Gut .2004; 53:1374-84.
7. Woo HY, Park DI, Park H, Kim MK, Kim DH, Kim IS, et al. Dual-priming oligonucleotide-based multiplex PCR for the detection of Helicobacter pylori and determination of clarithro-mycin resistance with gastric biopsy specimens. Helicobacter. 2009; 14:22–28.
8. Souod N, Kargar M, Doosti A, Ranjbar R and Sarshar M. Genetic Analysis of cagA and vacA Genes in Helicobacter Pylori Isolates and Their Relationship with Gastroduodenal Diseases in the West of Iran. Iran Red Cres Med J. 2013; 15(5): 371-5.
9. Anastiosis R, Goritsas C, Papamihail C, Trigidou R, Garzonis P, Ferti A. Helicobacter pylori infection in diabetic patients: prevalence and endoscopic findings. Eur J Intern Med. 2002; 13: 376-9
10. Ramis IB , Pinho de Moraes E , Fernandes MS, Mendoza-Sassi R, Rodrigues O, Juliano CRV, et al. Evaluation Of Diagnostic Methods For The Detection Of Helicobacter Pylori In Gastric Biopsy Specimens Of Dyspeptic Patients. Brazilian Journal of Microbiology. 2012 : 903-908.
11. Lage AP, Godfroid E, Fauconnier A, Burette A, Butzler JP, Bollen A, et al. Diagnosis of Helicobacter pylori infection by PCR: comparison with other invasive techniques, detection of cagA gene in gastric biopsy specimens. J Clin Microbiol. 1995; 33: 2752-2756.
12. Abdollahi H, Savari M, Zahedi MJ, Moghadam SD, Abasi MH. Detection of A2142C, A2142G, and A2143G Mutations in 23S rRNA Gene Conferring Resistance to Clarithromycin among Helicobacter pylori Isolates in Kerman, Iran. Iran J Med Sci. 2011; 36 (2):104 -110.
13. Alboraie M, Elhossary W. Helicobacter pylori infection in Egypt; a very common disease with low evidence based clinical practice. Arab Journal of Gastroenterology 19 (2018) : 49.

14. Malik GM, Mubarik M and Kadla SA. Helicobacter pylori Infection in Endoscopic Biopsy Specimens of Gastric Antrum: Laboratory Diagnosis and Comparative Efficacy of Three Diagnostic Tests. Diagn. Ther. Endosc. 1999; 6 (1): 25-29.
15. Patel SK, Pratap CB, Jain AK, Gulati AK and Nath G . Diagnosis of Helicobacter pylori: What should be the gold standard? World J Gastroenterol. 2014; 20(36): 12847-12859.
16. Ghaith D, Elzahry M, Mostafa G, Mostafa S, Elsherif R, Ramzy I. Mutations affecting domain V of the 23SrRNA gene in Helicobacter pylori from Cairo, Egypt. J Chemother. 2016; 28:367-70.
17. El-Masry EA, Melake NA , Salama AA, Makled AF. Detection of A2142G and A2143G Substitutions among Clarithro-mycin resistant Helicobacter pylori Strains Isolated from Egyptian Patients. British Microbiology Research Journal. 2016; 15(2): 1-9
18. Khalifehgholi M, Shamsipour F, Ajhdarkosh H, Daryani NE, Pourmand MR, Hosseini M, et al. Comparison of five diagnostic methods for Helicobacter pylori . Iran. J. Microbiol. 2013; 5(4): 396-401.
19. Khashei R, Dara M, Bazarg-ani A, Lankarani KB, Taghavi A, Moeini M, et al .High rate of A2142G point mutation associated with clarithromycin resistance among Iranian Helicobacter pylori clinical isolates. APMIS. 2016; 124: 787–793.
20. Ramzy I, Elgarem H, Hamza I, Ghaith D, Elbaz T, Elhosary W, et al. Genetic mutations affecting the first line eradication therapy of Helicobacter pylori infected Egyptian patients. Rev Inst Med Trop Sao Paulo. 2016; 2916: 58- 88.
21. Zaki ME , Sherif MD, Ali MA, Shehta A, Megahed A, Alsayed MA, et al. Molecular Study of Primary Clarithromycin Resistant Helicobacter pylori strains from Egyptian Centre. Int.J.Curr. Microbiol. App.Sci . 2016; 5(1): 165-173.
22. Diab M, El-Shenawy A, El-Ghannam M, Salem D, Abdel-nasser M, Shaheen M, et al. Detection of antimicrobial resistance genes of Helicobacter pylori strains to clarithromycin, metronidazole, amoxicillin and tetracycline among Egyptian patients . The Egyptian Journal of Medical Human Genetics (2018), https://doi.org/10.1016/j.ejmhg.2018.01.004
23. De Francesco V, Margiotta M, Zullo A, Hassan C, Giorgio F, Burattini O, et al . Prevalence of primary clarithromycin resistance in Helicobacter pylori strains over a 15 year period in Italy. J Antimicrob Chemother. 2007; 59: 783–785.
24. Abadi AT, Taghvaei T, Ghasem-zadeh A and Mobarez AM. High frequency of A2143G mutation in clarithromycin-resistant Helico-bacter pylori isolates recovered from dyspeptic patients in Iran. Saudi J Gastroenterol. 2011; 17: 396-9.