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ORIGINAL ARTICLE

Relation between *Helicobacter pylori* CagA gene Status and Antibiotic Resistance Pattern in Peptic Ulcer Patients

Azza Z Labeeb* and Amira H EL-Khyat
Department of Medical Microbiology and Immunology, Faculty of Medicine, Menoufia University, Egypt

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****Corresponding Author:***
Azza Z.Labeeb
Department of Medical Microbiology and Immunology, Faculty of Medicine, Menoufia University, Egypt.
Tel.: 01006399899
mhodmaghraby@yahoo.com

ABSTRACT

Background*: Helicobacter pylori (H. pylori) associated peptic ulcer represents a therapeutic challenge with almost no regimen having achieved 100% eradication due to the emergence of antibiotic resistance. H. pylori virulence genes not only contribute in its pathogenesis but also have an influence on antibiotic resistance. Hence, the study of virulence genes effect on the acquisition of drug resistance may be an avenue to explore how to improve H. pylori eradication rates.* ***Objectives:*** *To estimate the prevalence of H. pylori cytotoxin-associated gene A (CagA) positive genotype among peptic ulcer(PU) patients, and to evaluate if there is an association between CagA virulence gene status and antibiotic resistance patterns in H. pylori strains.* ***Methodology:*** *Gastric biopsy specimens were collected from 54 enrolled patients with suspected peptic ulcer disease. Eighteen gastric biopsy specimens yielded H. pylori growth which were confirmed microscopically and by biochemical traits. Antimicrobial susceptibility patterns of H. pylori isolates were determined by disc diffusion and agar dilution methods. CagA was determined using PCR.* ***Results:*** *H. pylori CagA positive isolates were more significantly presented among peptic ulcer patients 13/18 (72.2%) compared to CagA negative isolates 5/18 (27.8%). High rates of antibiotic resistance against the first-line regimen of H. pylori associated PU were revealed with resistance rates of 100%, 61.1%, and 50% to metronidazole, amoxicillin and clarithromycin respectively, however (14/18; 87.8%) of H. pylori isolates were susceptible to ciprofloxacin and tetracycline. H. pylori CagA negative strains were associated with high levels of resistance to ciprofloxacin, clarithromycin, amoxicillin and tetracycline compared to CagA positive isolates.* ***Conclusion:*** *this work elucidated the high prevalence of H. pylori CagA positive genotype among Egyptian patients. The high level of resistance against the first line regimen of H. pylori associated peptic ulcer is alarming. Ciprofloxacin and tetracycline, both drugs can be the best choice in treating peptic ulcer patients. Absence of CagA gene in H. pylori was associated with high degrees of resistance and the presence of CagA gene be a predictor marker for H. pylori successful eradication .*

INTRODUCTION

H. pylori is a spiral–shaped, Gram negative bacterium. It is an opportunistic pathogen, however, it can infect more than 50% of the world's population because of its high colonization rate and persistent nature in gastric mucosa. Fortunately, colonization with *H. pylori* is silent in most cases but overt damage of mucosa can occur causing a variety of gastroduodenal diseases ranging from gastritis and peptic ulcer disease (PUD) to gastric cancer in some cases[1]

H. pylori is usually described as a poor man's gut bacterium, hence, infection with *H. pylori* is very high (up to 80%) in developing countries[2]. Recently, observations were reported regarding the risk of developing *H. pylori* associated diseases which is usually depends on a combination of different factors such as; virulence of *H. pylori*, host's defense mechanisms and environmental factors [3].

H. pylori harbored many virulence coding genes such as urease (UreA), cytotoxin associated gene A (CagA) and vacuolating toxin (VacA). However, it is believed that CagA gene is a key marker in the pathogenesis of *H. pylori* as it allows colonizing the gastric mucosa and encodes a protein that is associated with an increase in intensity of gastric inflammation and consequently, with severe clinical outcomes[1].

Interestingly, the prevalence of *H. pylori* CagA genotype associated diseases is totally different among geographic regions. A recent report from Egypt indicates that more than 70% of the population is infected with *H. pylori* , particularly, CagA genotype associated gastritis and gastric cancer[4] .

Management of *H. pylori* induced diseases basically aims to treat peptic ulcer and limit the complication of developing cancer. Up till now, *H. pylori* treatment is recommended by proton pump inhibitors combined with

two antimicrobial agents e.g. amoxicillin, clarithromycin or metronidazole[1] .

Growing antibiotic resistance in *H. pylori* is a global issue as it may cause failure in strategies of treatment plane. Antimicrobial resistance profile differs within patient groups according to the geographic region, age, sex, and the presence of other infections[5].

This study was conducted to estimate the prevalence of *H. pylori* CagA positive genotype among peptic ulcer patients, and to evaluate if there is association between CagA virulence factor status and antibiotic resistance pattern in *H. pylori* isolates to attain a clinical utility from such work.

METHODOLOGY

This is a cross sectional study conducted over a period of 12 months, in collaboration between Internal Medicine Endoscopy Unit and Microbiology department, Faculty of Medicine, Menoufia University. This study included 54 participants, they were scheduled for endoscopy based on various symptoms indicating gastrointestinal disorders such as, recurrent epigastric pain, nausea , bloody vomit or black stool. Their age ranged from 21 to 51 years old .A written informed consent was obtained from each patient before inclusion in the study. Selected cases were subjected to complete history taking and diagnostic work-up including complete clinical and laboratory assessment. The study protocol was approved by the local ethics committee of the Menoufia University.

Inclusion criteria:

Patients were eligible for this study if they were diagnosed as peptic ulcer (PU) according to their endoscopy reports and were confirmed for *H. pylori* infection by positive rapid urease test, positive culture and biochemical reactions.

Exclusion criteria:

Any participant had a risk to develop complications after endoscopy were excluded.

Samples collection:

At least six gastric biopsies; three antrum and three corpus biopsy specimens were collected for each patient. Of these, one antrum and one corpus biopsies were used for screening of *H. pylori* by rapid urease test. The other four biopsies were transported immediately to the Microbiology laboratory in sterile tubes containing Brain Heart Infusion broth (BHI, Oxoid) to be used for *H. pylori* culture.

Culture and identification of H. pylori:

Biopsy specimens were manually grinded .Cultures were performed on Campylobacter Skirrow's selective medium (Oxoid), supplemented with 10% blood, and incubated under microaerophilic conditions using Campylobacter gas kit (Oxoid) for 3-5 days . *H. pylori* isolates were identified by their specific colony morphology (dew drop appearance), microscopically (red curved or straight Gram negative rods) and positive biochemical profiles (urease, catalase and oxidase tests positive) .Strains were maintained at −80◦C in BHI broth with 30% glycerol until DNA extraction[5].

Antibiotic susceptibility testing by disc diffusion method:

All *H. pylori* isolates were screened for resistance against five antibiotics, clarithromycin (CLA), ciprofloxacin (CIP), amoxicillin (AMX), tetracycline (TET), and metronidazole (MTZ), using disk diffusion method according to the Clinical and Laboratory Standard Institute (CLSI) guidelines[6]. Muller Hinton's agar plates supplemented with 10% blood were inoculated by *H. pylori* culture suspension with a turbidity equivalent to 2 McFarland standard for each isolate. Zone diameters were read after 24–48 h of incubation under microaerophilic conditions and were interpreted according to the CLSI guidelines[6] and previous literatures[7].

MIC determination by agar dilution method:

Agar dilution method was performed according to CLSI[6]. The final concentrations of tetracycline, ciprofloxacin, and amoxicillin ranged from 0.016 to 64 µg/mL, while metronidazole and clarithromycin concentrations ranged from 0.016 to 256 µg/mL. The MICs values were the lowest antibiotic concentration that completely inhibited visible growth of the bacteria. Clarithromycin MICs were interpreted according to CLSI guidelines (MIC ≥ 1µg/ml, resistant and MIC =0.25 µg/ml, susceptible) [6]. The other four antibiotics do not have CLSI breakpoints so were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST)[8] , and their resistance breakpoints are, metronidazole > 8 µg/ml, tetracyclin > 1 µg/ml, amoxycillin > 0.12 µg/ml and ciprofloxacin >1µg/ml. The plates were incubated at 37◦C for 3-5 days under microaerophilic conditions [5].

DNA extraction and CagA gene amplification:

Genomic DNA was extracted from all *H.pylori* stains using Quick- DNA TM Miniprep Kit, USA according to the manufacturer's guidelines, primers[2,9] used in this study were listed in table (1). For screening of CagA gene reaction mixture contained 1 µL of primer, 1 µL of genomic DNA, 12.5 µL PCR Master Mix, and add H2O to a total volume of 25 µL. Thermocycling conditions were as follows; pre-incubation cycle at 94°C for 2 minutes followed by 35 cycles at 95°C for 1 minute, 50 °C for 1 minute, and 72°C for 1 minute, with a final extension step of 72°C for 5 minutes[9]

Statistical analysis

Categorical variables are described as n (%). For comparative test the Pearson Chi‑square test or Fisher's exact test were used as appropriate. Statistical analysis was done using the SPSS‑17 software. *P* <.05 was considered significant.

Table1: Primers used in the study

Target gene	Sequence (5'-3')	PCR Products
CagA- F CagA-R	ATAATGCTA AATTA GA CAACTTGAGCGA AGAAACAAAAGC AATACGATCA TT C	298 bp

RESULTS

Among 54 collected gastric biopsy specimens, *H. pylori* infection was found in 18/54 (33.3%) patients. Eighteen peptic ulcer patients were enrolled in this study, 11 males and 7 females, ranging in age from 21 to 51 years. As per our selection criteria, the eighteen gastric biopsy specimens, which were collected from enrolled candidates, yielded *H. pylori* growth that were confirmed microscopically and by biochemical traits. *H. pylori* CagA positive isolates were more significantly presented among peptic ulcer patients 13/18 (72.2%) compared to CagA negative isolates 5/18 (27.8%). The demographic and clinical data of *H. pylori* infected patients were shown in table (2). There was no difference between patients infected with CagA positive and CagA negative *H. pylori* isolates regarding gender or history of drug intake, however, association of clinical manifestations among patients infected with *H. pylori* CagA positive genotype strains was significantly higher compared to CagA negative isolates.

Table 2: Demographic and clinical presentation of patients infected with cagA positive and cagA negative *H. pylori* strains.

Studied variables	Total *H. pylori* strains n=18		CagA positive strains *H. pylori* n=13		CagA negative *H. pylori* strains n=5		χ^2	P value
	n	%	n	%	n	%		
n(%)	18	100	13	72.2	5	27.8		
Sex								
Male	11	61.1	8	61.5	3	60	0.302	0.583
Female	7	38.9	5	38.5	2	40		
Associated symptoms								
Pain	8	44.4	6	46.2	2	40		
Nausea	2	11.1	2	15.3	0	0		
Haematemesis	3	16.6	3	23	0	0	21.7	**0.012***
Black stool	1	5.5	1	7.7	0	0		
History of antibiotics intake								
Yes	12	66.7	10	77	2	40	2.21	0.136
No	6	33.3	3	33	3	60		

Significant

Table 3: Antimicrobials susceptibility test results of *H. pylori* strains (18) by agar dilution and disk-diffusion methods.

Method		Metronidazole	Amoxicillin	Ciprofloxacin	Clarithromycin	Tetracycline
Agar Dilution	**MIC range** (μg/mL)	8-256	0.016 -0.064	1-32	1-256	1-64
	Resistant n(%)	18(100%)	11(61.1%)	4(22.2%)	9(50%)	4(22.2%)
	Sensitive n(%)	0	7(38.9%)	14(87.8%)	9(50%)	14(87.8%)
Disk-diffusion	**Resistant n(%)**	18(100%)	10 (55.6%)	4(22.2%)	7(38%)	5(27.8%)
	Sensitive n(%)	0	8(44.4%)	14(87.8%)	11(61.2%)	13(72.2%)

Antimicrobial susceptibility patterns of 18 *H. pylori* strains were determined by disc diffusion and agar dilution methods .The overall resistance rates against the five tested antibiotics, metronidazole, amoxicillin, ciprofloxacin, clarithromycin, and tetracycline by both agar dilution and disk-diffusion methods are shown in table (3) and figure (1). *H. pylori* strains showed 100% resistance against metronidazole by both agar dilution and disk-diffusion methods. The other antibiotics showed variable results concerning

disk diffusion and agar dilution methods. About 11/18 (61.1%) and 9/18 (50%) of *H. pylori* strains were resistant to amoxicillin and clarithromycin by agar dilution method versus 10/18 (55.6%) and 7/18 (38%) resistance rates by disk diffusion method. However, most isolates were susceptible to ciprofloxacin 14/18 (87.8%) by both methods) and tetracycline 14/18 (87.8%) by agar dilution method and 13/18 (72.2%) by disk diffusion method), both drugs were highly effective in-vitro against *H. pylori* isolates .

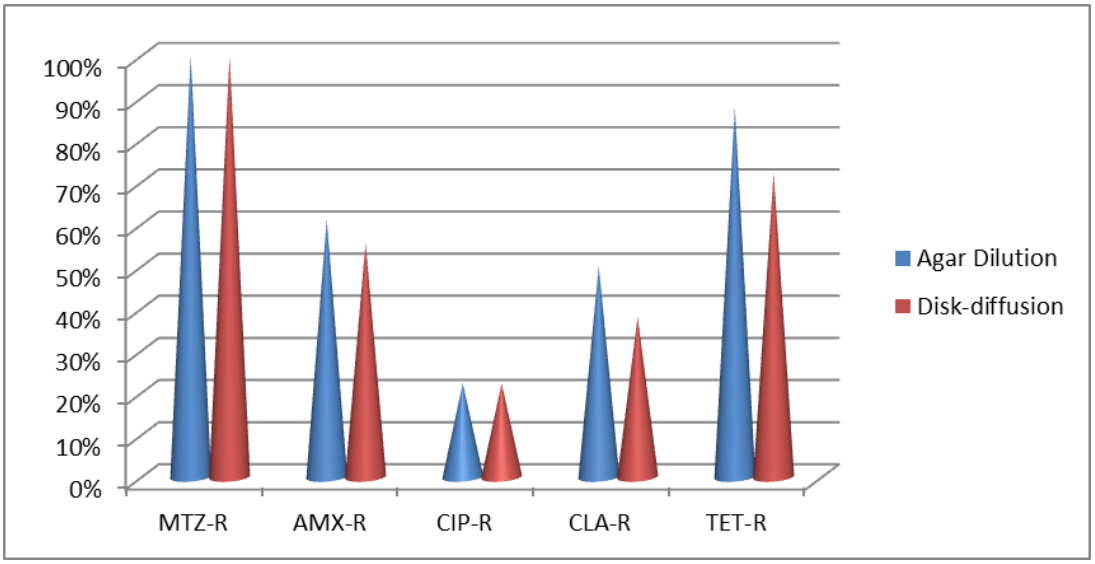

Fig. 1: Antibiotic-resistance patterns of total *H. pylori* isolates (18) using disk diffusion and agar dilution methods. . MTZ-R, metronidazole resistant; AMX-R, amoxicillin resistant; CLA-R, clarithromycin resistant; CIP-R, ciprofloxacin resistant ;TET-R, tetracycline resistant.

Table 4: Comparison of antibiotic sensitivity patterns in *H. pylori* CagA positive genotype and CagA negative genotype isolates according to MIC test results .

Antimicrobial agent / Disk content (ug)	*Cag A positive H. pylori* isolates (n=13)				*Cag A* negative *H. pylori* isolates (n=5)				Total *H. pylori* isolates (n=18)				P value
	Sensitive		Resistant		Sensitive		Resistant		Sensitive		Resistant		
	n	%	n	%	n	%	n	%	n	%	n	%	
Metronidazole (5ug)	0	0	13	100	0	0	5	100	0	0	18	100	--
Amoxicillin(10ug)	6	46.2	7	53.8	1	20	4	80	7	38.9	11	61.1	0.952
Ciprofloxacin(5ug)	12	92.3	1	7.7	2	40	3	60	14	87.8	4	22.2	**0.0168***
Clarithromycin (15ug)	7	53.8	6	46.2	2	40	3	60	9	50	9	50	0.598
Tetracycline (30ug)	11	84.6	2	15.4	3	60	2	40	14	87.8	4	22.2	0.2605

**** Significant***

Antimicrobial sensitivity patterns of *H. pylori* isolates with respect to its relation with CagA gene status are listed in table (4) and figure (2).There was no difference between CagA positive and CagA negative *H. pylori* isolates regarding metronidazole resistance (100% for both) .

However, analysis of the other antibiotics sensitivity patterns indicates lower resistance rates of amoxicillin 7/13 (53.8%), clarithromycin 6/13 (46.2%), and tetracycline 2/13 (15.4%) in CagA positive compared with CagA *negative isolates* (with 80% , 60% ,and 40% resistance respectively). Also, we found a significant higher ciprofloxacin resistance among CagA negative *H. pylori* isolates 3/5 (60%) compared with CagA positive isolates 1/13 (7.7%) (P< 0.05).

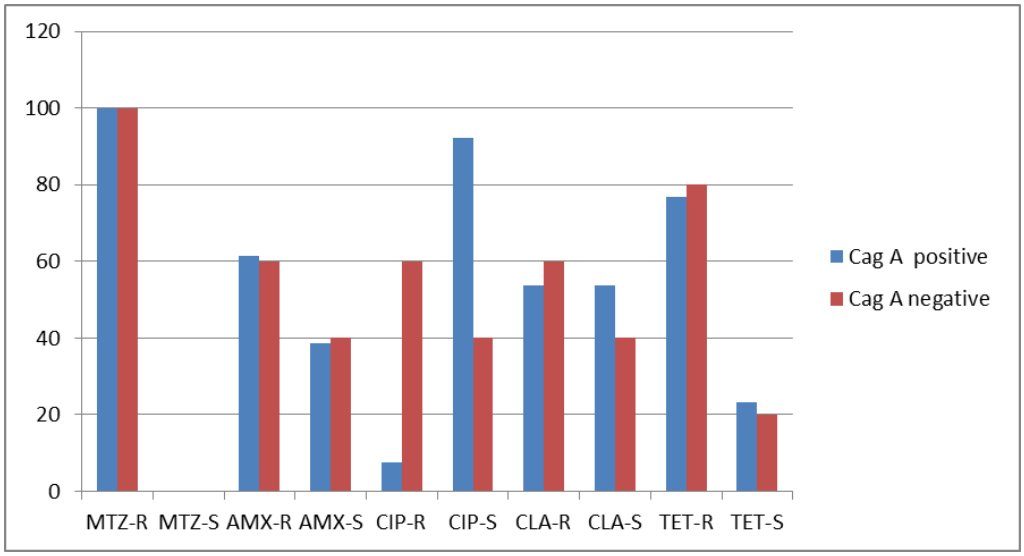

Fig. 2: Comparing antibiotic sensitivity patterns in *Cag*A positive and CagA negative *H. pylori* isolates according to MIC test results. MTZ-R, metronidazole resistant; MTZ-S, metronidazole sensitive; AMX-R, amoxicillin resistant; AMX-S, amoxicillin sensitive; CLA-R, clarithromycin resistant; CLA-S, clarithromycin sensitive ;CIP-R, ciprofloxacin resistant; CIP-S, ciprofloxacin sensitive; TET-R, tetracycline resistant; TET-S, tetracycline sensitive.

Table 5: CagA positive and CagA negative *H.pylori* antibiotics susceptibility patternsin relation to history of antibiotics intake in PU infected patients.

Antimicrobial agents	Patients with prior antibiotics exposure					
	Cag A *positive* *H. pylori* isolates (n=10/13)		Cag A negative *H. pylori* isolates (n=2/5)		Total *H. pylori* isolates (n =12/18)	
	n	%	n	%	n	%
Metronidazole						
Resistant	10	100	2	100	12	100
Sensitive	0	0	0	0	0	0
Amoxicillin						
Resistant	8	80	2	100	10	83.3
Sensitive	2	20	0	0	2	16.7
Ciprofloxacin						
Resistant	0	0	2	100	2	16.7
Sensitive	0	0	0	0	0	0
Clarithromycin						
Resistant	4	40	2	100	6	50
Sensitive	6	60	0	0	6	50
Tetracycline						
Resistant	0	0	2	100	2	16.7
Sensitive	0	0	0	0	0	0

In total 10/13 (77%) of patients infected with CagA positive, and 2/5 (40%) of patients infected with CagA negative *H.pylori* strains had a history of prior antibiotics exposure. 100% resistance against all drugs was observed in all patients infected with CagA negative isolates with history of antibiotic exposure, compared to 100% metronidazole, 80% amoxicillin, and 40% clarithromycin resistance in CagA positive strains, but none of those patients showed history of ciprofloxacin or tetracycline intake (Table 5).

DISCUSSION

H. pylori infection is one of the most prevalent chronic bacterial disease as it affects more than half of the population globally[10]. Increasing reports of *H. pylori* associated peptic ulcer treatment failure necessitate

surveillance studies to analyze the trend of drug resistance especially in developing countries where multidrug resistance is quite common in most bacterial species[11] .Genotypic differences of *H. pylori* directly influence the pathogenesis of infection. Such effects have been widely evidenced in *H. pylori* CagA genotype, however the exact association between virulence genes and antibiotic resistance remains elusive[10].

H. pylori culture detection is a highly specific diagnostic method, and has a great advantage of allowing performance of antibiotic sensitivity patterns. This study included 54 participants with gastrointestinal disorders, among them 18/54 (33.3%) patients were diagnosed as *H. pylori* associated PUD using phenotypic detection methods (culture and biochemical reactions).In this work, *H. pylori* prevalence rate in peptic ulcer patients, using conventional culture, was 33.3%. Our finding showed a comparable prevalence rate to those reported by, Fathi et al., from Egypt[12]. The prevalence of *H. pylori* had been reported to differ among countries, and high infection rates being associated with bad hygiene practices, low socioeconomic status and high densities of living[10].

In the present study, a high incidence of *H. pylori* *C*agA genotype in PU patients was identified ,where almost 13/ 18 (72.2%) of isolates were positive *C*agA. This result was comparable to those reported from other studies, such as (73.3%)[9], and (66%) in Egypt[12], (67.6%) in Tunisia[13] , and (76.9%) in Saudi Arabia[2] . However in other studies, CagA gene was even lower in *H. pylori* strains, as in Egypt 50% [13], and Middle East countries, as Jordan (26%)[14] and Kuwait (40%)[15].

In our study the antimicrobial sensitivity of 18 *H. pylori* isolates was determined against the most commonly used antibiotics for *H. pylori* eradication (metronidazole, tetracycline, clarithromycin, amoxicillin and ciprofloxacin) using the disc diffusion and agar dilution method. High rates of antibiotic resistance against the first-line regimen of *H. pylori* associated PU were revealed with resistance rates of 100%, 61.1%, and 50% to metronidazole, amoxicillin and clarithromycin respectively. These results are in line with those reported by Fathi et al from Egypt, with resistance rates of 100 % for metronidazole and 50% for clarithromycin[5]. Metronidazole resistance is high in most developing countries (>80%)[10]. Khan and his colleagues from Pakistan reported a similar antibiotic susceptibility profile of *H. pylori* strains, with resistance rate of 100% for metronidazole, 74% for amoxicillin and 48% for clarithromycin[11]. However, lower metronidazole resistance of *H. pylori* isolates was reported from Middle Eastern countries, such as Saudi Arabia78%[2], Bahrain 57%[16], and United Arab Emirates 62%[16].

However, in our study, (87.8%) of *H. pylori* isolates were susceptible to ciprofloxacin and tetracycline, both drugs can be the best choice in treating Egyptian patients infected with *H. pylori* due to their potent activity and excellent susceptibility (87.8%).These findings are comparable to those reported by Al-Eraky et al In Egypt, with 83% and 75% susceptibility of *H. pylori* isolates to ciprofloxacin and tetracycline respectively[4]. Reported resistance to ciprofloxacin is very low worldwide such as, 14.8% [17], 20% [13], and 18.5%[10]. Ciprofloxacin is highly active in eradication of *H. pylori*, however, its intense side effects hamper its wide use[11]. Ciprofloxacin low resistance rates could be explained by infrequent mutations of quinolone resistant genes[5].

H. pylori genome screening is necessary to determine if acquisition of antibiotic resistance is a phenomenon for optimizing virulence. It will be noteworthy to explore the correlation of antibiotic resistance with virulent genotypes[1].

In our work, The differential prevalence of resistance patterns in CagA positive and CagA negative *H. pylori* isolates, indicated that CagA gene has a limited role in acquired antibiotic resistance, as only a significant higher ciprofloxacin resistance in CagA negative (60%) compared to CagA positive (7.7%) isolates was identified.

In agreement with our results, Khan et al[10] reported a possible link between *H. pylori* CagA gene and acquired ciprofloxacin resistance, as its resistance rate was lower in CagA positive (11%) compared with in CagA negative (22%) isolates. The explanation of this result is that CagA negative strains have the ability to acquire antibiotic resistance through maintaining their high mutations rate especially under selective pressure conferred by high ciprofloxacin concentration whereas a decline of resistant mutants observed in CagA positive strains[1].

Also, in this study less virulent CagA negative isolates showed higher resistance to amoxicillin , clarithromycin , and tetracycline compared with CagA positive isolates, these results are matched with[18,19] . So, our work results evidently showed that absence of CagA gene may help in the acquisition of drug resistance in *H. pylori* isolates .

These findings can be explained by the ability of virulent CagA positive strains to cause intense inflammation with increased blood supply and antibiotics absorbtion at that site and eventually lead to better *H. pylori* eradication rate, with a limited contribution of CagA gene[1,10] . On the other side, less virulent strains are less immunogenic, less antibiotics will reach infected areas in the stomach , hence among less virulent bacterial population selection of resistant one will take place . It has been shown that a CagA negative isolates tend to acquire drug resistance in vitro[20].

In present study, 100% resistance of tested antibiotics was observed in all patients infected with

CagA negative isolates and had history of antibiotic intake, compared to, 100% metronidazole, 80% amoxicillin, and 40% clarithromycin resistance in CagA positive strains. High resistance rates among *H. pylori* isolates obtained from patients with positive history of antibiotic intake were previously reported[21].

CONCLUSION

In conclusion, this work elucidated the high prevalence of *H. pylori* CagA positive genotype among Egyptian patients which may be involved in high degree of inflammation and may predispose to gastric cancer. The high resistance rates against drugs used in the first line regimen of *H. pylori* associated peptic ulcer (metronidazole, amoxicillin, and clarithromycin) is alarming. In Egypt, the need for continuous surveillance for *H. pylori* drug resistance pattern is mandatory in planning for effective treatment strategies. Less virulent, *H. pylori* CagA negative strains were associated with high degrees of antibiotic resistance ,hence the presence of CagA gene may be a predictor marker for *H. pylori* successful eradication .

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Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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