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ORIGINAL ARTICLE

Frequency of *rdx A* Deletion Mutation Conferring Metronidazole Resistance in *Helicobacter pylori*

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ABSTRACT

Background: *Metronidazole is one of the antimicrobial drugs that can be used in combination with other drugs for eradication of Helicobacter pylori (H. pylori).Unfortunately, metronidazole resistance in H. plori is an increasing health problem which may be attributed to inactivation of many genes as rdx A gene.* ***Objective:*** *To determine the frequency of rdx A deletion mutation in H. pylori detected in infected patients attending at the Gastroenterology Unit, Zagazig University Hospitals.* ***Methodology:*** *Two gastric biopsies were taken from each enrolled patient by endoscopy. H.pylori detection was done by rapid urease test and polymerase chain reaction (PCR) amplification of 16S rRNA gene. Deletion mutation in rdx A gene was detected by conventional PCR.* ***Results****: Out of 134 doubled gastric biopsies obtained from 134 patients, 52.2% were positive for H. pylori. Epigastric pain, vomiting and gastritis were significantly associated with detection of H. pylori infection ($p < 0.05$). Deletion mutation of rdx A gene was detected in 28.6% of H. pylori positive specimens obtained from infected patients.* ***Conclusion:*** *Deletion mutation of rdx A gene is a frequent determinant of rdx A inactivation conferring metronidazole resistance among H. pylori.*

INTRODUCTION

Helicobacter pylori (*H. pylori*) are a spiral, microaerophilic, Gram-negative bacterium that colonizes the gastric mucosa. In developing countries, the prevalence of infection is recorded to be very high with up to 90% of the population being infected[1]. Infected patients may develop gastritis, doudenal ulcer, gastric ulcer, gastric cancer and in some occasions, mucosa-associated lymphoid tissue lymphoma[2].

In Egypt, metronidazole, a proton pump inhibitor and either clarithromycin or amoxicillin are used as a triple standard eradication therapy for *H. pylori*[3].

Metronidazole resistance had been frequently detected among isolated *H. pylori* with a frequency ranging from10% to 90% or even more and it varies according to geographic area and patient groups[4].

Owing to the troublesome procedures needed for isolating *H. pylori* and determining their in vitro antibiotic susceptibility, metronidazole resistance, along with the resistance to other antimicrobials, is preferably detected at the molecular level. So, many studies have investigated the detection of different genetic mutations that are known to confer known resistance mechanisms[2,3,5].

Goodwin and his colleagues[6] were the first researchers to show that metronidazole resistance might be attributed to mutational inactivation of *rdx A* gene which is attributed mainly to a 200 bp deletion mutation and to less extent to other mutations such as insertion, frameshift and missense mutations[7].Other mechanisms of metronidazole resistance included *rdx A* gene inactivation due to the insertion of Mini-IS605 transposon and other genes inactivation such as the *frx A* gene either alone or combined with inactivation of *rdx A* gene[8].

To achieve an antimicrobial action, metronidazole needs an activation step that necessitates reduction by the microbe redox system; essentially by an oxygen-insensitive (type I) NADPH nitroreductase which is encoded by *rdx A* gene. Consequently, inactivation mutation of this gene mediates a resistance to metronidazole[4, 9].

As limited data exists concerning the frequency *rdx A* deletion mutation, this study was done to detect the frequency of *rdx A* gene deletion mutation in *H. pylori* from patients attending Zagazig University Hospitals.

METHODOLOGY

Study design:

This cross-sectional study was carried out over 12 months from December 2018 to December 2019 at the Medical Microbiology and Immunology Department, Faculty of Medicine, and Gastrointestinal Endoscopy Units at Zagazig University Hospitals.

***Ethical consideration*:**

The institutional review board (IRB), Zagazig Faculty of Medicine approved this work, and consents were taken from all study participants.

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Patients:

This study enrolled134 patients; including 63 males and 71 females with their age range from 21-67years. They were recommended for endoscopy after complaining of upper GIT symptoms (epigastric pain, vomiting or dyspepsia). ***Exclusion criteria:***

Patients who refused to be enrolled in this study, patients receiving treatment for *H. pylori* during a month prior to the endoscopy, patients with any contraindication or had a risk to develop complication after endoscopy were excluded from the study.

Samples collection:

A disinfected endoscope (GIF XQ230; Olympus, Center Valley, PA) was used to obtain two antral gastric biopsy specimens from each patient. Rapid urease test (RUT) was used to examine first biopsy and the second one was placed in 0.1 ml saline and stored at -20˚C till used for DNA extraction.

Identification of *H. pylori* infection:

This was performed by rapid urease test (RUT) and molecular amplification of *H. pylori 16S rRNA* gene using polymerase chain reaction (PCR).

Rapid urease test:

This was done by placing the first gastric biopsy on a commercial paper RUT **(HelicotecUT®Plus, strong Biotech Corporation, Taiwan)**. Color changes were recorded within one hour. Positive result was recorded if color changed to pink or red while negative result was considered if color remained yellow.

PCR tests:

DNA was extracted from the second gastric biopsies using theQIAamp® DNA Mini kit, QIAGEN, Germany, according to the manufacturer's instructions and stored at -20˚C until amplification was performed. Detection of *H. Pylori 16S rRNA* by conventional PCR was followed by amplification of *rdx A* for molecular detection of metronidazole resistance.

Detection of H. pylori 16S rRNA:

PCR was performed using Veriti ®96-Well thermal cycler (Applied Biosystems, Singapore).This was performed according to Ramzy and his colleagues[5], in a final volume of 20 µL including, 1µL of the template DNA, 1 µL of each of the oligonucleotide primers and 17µL of sterile deionized water were added to each PCR bead of Maxime PCR PreMix Beads (iNtron, Certified Company, Germany)**.** Primers and PCR conditions are listed in tables i and ii.

Detection of metronidazole resistance:

DNA extracts that were shown positive for *H. pylori 16S rRNA* were further subjected to amplification of *rdx A* gene. PCR was performed using the same previous loading volumes in *16S rRNA* gene amplification with specific primers and cycling conditions listed in tables i & ii. Amplified products were visualized on 2% agarose gel under UV light.

Table i: Primers sequence and amplicon sizes of PCR tests

Gene	Sequence	Size	Gene	References number
Hp-F *Hp-R*	CTGGAGAGACTAAGCCCTCC ATTACTGACGCTGATTGTGC	110 bp	*H. pylori16SrRNA*	10
rdxA-F *rdxA-R*	AATTTGAGCATGGGGCAGA GAAACGCTTGAAAACACCCCT	850bp 650bp	Wild *rdx A* gene Mutated *rdx A* gene	11

Table ii: PCR conditions used for amplification of *16S rRNA and rdx A* genes:

Step	Amplification of *H. pylori 16S rRNA* gene			Amplification of *rdx A* gene		
	Temperature	Time	Cycles number	Temperature	Time	Cycles number
Initial denaturation	95 ˚C	5 min	1	94˚C	5 min	1
Denaturation	95˚C	1min	35	94˚C	1min	30
Annealing	60˚C	1 min	35	55˚C	1 min	30
Extension	72˚C	1 min	35	72˚C	1 min	30
Final extension	72˚C	10 min	1	72˚C	10 min	1

Statistical analysis of data:

Statistical packages (EPI-info Version 6.04 and SPSS Version 20 inc. Chicago, USA) were used to analyze collected data. A p-value<0.05 was considered to be statistically significant at 95% confidence interval. Chi-square test was used to compare proportions.

RESULTS

This study enrolled 134 patients including, 63 males and 71 females with their age ranging from 21 to 67 years (mean 46.7±14.9). More than half of them were suffering from epigastric pain as well as gastritis was the main endoscopic finding among them (table 1).

Out of 134 doubled gastric biopsies obtained from enrolled patients, 70 patients (52.2%) were positive for *H. pylori* by rapid urease and PCR. The demographic data, clinical manifestations and endoscopic findings in *H. pylori* infected patients, compared to those found to be not infected, are demonstrated in table 2. Epigastric pain and vomiting were significantly associated with *H. pylori* detection (p<0.001 & 0.008*, respectively).

Among positive *H. pylori* infected patients (n=70), *rdx A* deletion mutation was detected in 20 patients recording a frequency of 28.6% (table 3).

Table 1: Demographic character, clinical manifestation and Endoscopic findings

Variable	Mean±SD	Range
Age	46.7±14.9	21-67
	No.	**%**
Sex		
Male	63	47.0
Female	71	53.0
Clinical manifestation		
Epigastric pain	75	56.0
Heart burn	55	41.0
Haematemisis	12	9.0
Dyspepsia	28	20.9
Vomiting	35	26.1
Endoscopic findings		
Duodenal ulcer	25	18.7
Gastric ulcer	38	28.4
Gastritis	55	41.0
Gastric cancer	8	6.0
Total	134	100.0

Table 2: Demographic characters, clinical manifestations and endoscopic findings in *H. pylori* positive and negative patients:

Variable	*H. pylori* positive (No.70)		*H. pylori* negative (No.64)		X^2	P value
	No.	%	No.	%		
Age (years)						
<40	37	52.9	30	46.9	0.478	0.489
≥40	33	47.1	34	53.1		
Sex						
Male	30	42.9	33	51.6	1.017	0.313
Female	40	57.1	31	48.4		
Clinical manifestation						
Epigastric pain	55	78.6	15	23.4	40.732	<0.001**
Heart burn	30	42.9	25	39.1	0.198	0.655
Hematemesis	5	7.1	7	10.9	0.591	0.442
Dyspepsia	13	18.6	15	23.4	0.478	0.488
Vomiting	25	35.7	10	15.6	6.992	0.008*
Endoscopic findings						
Duodenal ulcer	19	27.1	6	9.4	6.954	0.008*
Gastric ulcer	20	28.6	18	28.1	0.003	0.954
Gastritis	40	57.1	15	23.4	15.696	<0.001**
Gastric cancer	5	7.1	3	4.7	0.359	0.549

X^2=Chi-square test.*Significant, **Highly significant

Table 3: Frequency of *rdx A* deletion mutation among positive *H. pylori* infected patients

***H. pylori* positive gastric specimens**	**No.**	**%**
***Rdx A* mutant type**	20	28.6
***Rdx A* wild type**	50	71.4
Total	70	100.0

DISCUSSION

Eradication of *H. pylori* has become difficult due to global increasing of its antimicrobial resistance. Continuous surveillance for detection of this resistance should be done to help physicians in selection of optimum eradication therapy[12, 13].

In the current study, *H. pylori* infection was detected in 52.2% of gastric biopsies of enrolled patients. This was in agreement with Sultan and his colleagues

(57.7%)[2]. Lower prevalence was detected in other studies as Abu-Taleb and her colleagues who reported a prevalence of 45.7%[14]. Also, Deeb and his colleagues reported that 44% of enrolled 400 school children in Al Qulubia were found to be infected with *H. pylori*[16]. Higher rate (64.6%) was detected in a study that investigated the effect of *H. pylori* infection on children growth by Galal and her colleagues[16]. This discrepancy in results could be attributed to the variance in sample size, age of enrolled patients and *H. pylori* detection methods used in different studies.

Concerning symptoms and endoscopic finding in *H. pylori* infected patients, epigastric pain, vomiting and gastritis were the most frequent and they were significantly associated with detection of *H. pylori* infection. This was in agreement with Ayana and her colleagues who reported that 61.1% of their dyspeptic patients had gastritis at endoscopic examination[17]. While, Srinivasan and his collogues reported that 66.5% of *H. pylori* infected patients were suffering from dyspepsia, and reported that gastritis was the commonest endoscopic finding followed by esophagitis and duodenal ulcer in their study at a rural area in India[18]. In a study of *H. pylori* infection and its correlation with diabetes mellitus, epigastric pain was the commonest complaint among patients as well as, duodenitis and oesophagitis were significantly associated with *H. pylori* infection[19]. On the other hand, Abbas and his colleagues found that nausea (25.5%), followed by gastric pain (24.5%) were the most frequent symptoms associated with infection among school children in Suddan[20].In Nepal, Shakya and his colleagues found that gastrointestinal bleeding was the only significantly associated symptom with *H. pylori* infection, although, heart burn was the most prevalent among patients[21].

Deletion mutation of *rdx A* gene was detected in nearly one third (28.6%) of *H. pylori* infected patients in the current study. This comes close to Diab and his colleagues who detected *rdx A* deletion mutation in 25% of studied *H. pylori*[3].Also, Abdollahi and his colleagues reported the prevalence of this type of mutation in 22.9% of resistant strains[22] and 25% of isolates were negative for wild *rdx A* gene by PCR in another study[8]. Confirming the major role of this mutation, another study showed that deletion mutation of *rdx A* gene was detected in seven of seventeen isolates (41.1%) exhibiting metronidazole resistance[23]. Zaki and his colleagues attributed the high metronidazole resistance reported in some studies to its wide use for gynecological, dental and parasitic infestations[24].On the other hand, lower frequency of deletion mutation was reported in other studies where mutant gene was detected only in 8% of studied *H. pylori* in Jordan by Abu-Qatouseh and his colleagues[25]. Also, 8.4% of strains had mutant gene by Amin and his colleagues in Iran[7].This discrepancy in results could be attributed to geographical difference in resistance pattern which reflects different antimicrobial policies followed in different countries.

CONCLUSION

Deletion mutation in *rdx A* gene is a frequent mutation detected in *H. pylori* infecting patients at Zagazig University Hospitals which warrants further studies on the contribution of this mutation on the clinical response of treated patients.

Recommendation: Metronidazole prescription should be rationalized with strict adherence to antimicrobial policy, continuous surveillance for detection of antimicrobial resistance and finding new alternatives to help physician for proper *H. pylori* eradication.

Limitations: The lack of studying of other determinants of metronidazole resistance as other mutations in *rdx A* and *frx A genes* which necessitates other molecular techniques such as real time PCR and gene sequencing, also the lack of in vitro susceptibility test for metronidazole**.**

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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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