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Real-time PCR detection of clarithromycin resistance genes in *Helicobacterpylori* from paraffin-embedded gastric biopsies in Gastric carcinoma patients.

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ABSTRACT

Helicobacter pylori (H. pylori) is a prevalent, worldwide, chronic infection. It remains an important factor linked to the development of peptic ulcer disease and gastric malignancy as MALT lymphoma. Clarithromycin is one of the antibiotics used for the treatment of H. pylori infections, and clarithromycin resistance is the most important factor when it comes to predicting eradication failure. Twenty, paraffin-embedded gastric biopsy specimens were obtained from the archives of Pathology Department Faculty of Medicine Ain Shams University, and were subjected to histopathological examination and Real time PCR for detection of clarithromycin resistance-associated gene mutations in H. pylori. All the 20 paraffin embedded gastric biopsy specimens showed PCR positivity for H. pylori, the mutated strains associated with resistance (A2142G orA2143G) were found in 12 specimens(60%),while the wild nonresistant strains were found in 8 specimens (40%). There was a significant correlation between the presence of resistant H. pylori strains and gastric erosions, while no significant correlation was found with all the other parameters of Sydney classification system. Meanwhile, all cases of MALT lymphoma and intestinal type gastric adenocarcinoma were positive for resistant strains, while all non-resistant strains were found among cases of diffuse type gastric carcinoma. However, the correlation between the resistant Strains and histologic type of gastric tumor was not statistically significant. Real-time PCR can be used as a rapid and reliable method for detection of clarithromycin-resistant H. pylori strains directly from paraffin-embedded gastric biopsy specimens. This might have a major impact on clinical management of H. pylori-associated gastritis and carcinoma.

Keywords: H. pylori; MALT lymphoma; Clarithromycin; PCR.

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INTRODUCTION

Helicobacter pylori (H. pylori) has been recognized as a causative factor in gastritis, duodenal and peptic ulcer, gastric carcinoma and mucosal associated lymphoid tissue (MALT) lymphoma. Its incidence has been increased in both developing and developed countries [1].Usually people infected with H.Pylori have no symptoms. Pathological changes associated with H.pylori gastritis include atrophic gastritis, intestinal metaplasia and dysplasia and are considered as predisposing factors for development of gastric adenocarcinoma[2,3] .Certain H pylori strains, that have cytotoxin associated antigen (cagA) were known also to increase the risk of gastric carcinoma [4,5].Moreover, in H.pylori -dependent gastric MALT lymphoma , the H. pylori-specific T cells raised in the reactive component of the classic germinal center, migrate to the marginal zone/tumor area providing non-cognate help to autoreactive neoplastic B cells, which may be involved in stimulation of CD40 and other surface receptors by soluble ligands and cytokines [6,7].

It has been reported that eradication of H.pylori has significantly decreased the risk of developing cancer in infected patients that did not have pre-malignant lesions. Accordingly, failure of treatment of H. pylori resistant strains forms a risk for oncogenesis[8].

Treatment of H.pylori is given not only for active peptic ulcer, but also for low-grade gastric MALT lymphoma, and after endoscopic resection of early gastric cancer [9].The best outcomes in the treatment of H. pylori infection have been obtained by treatment with triple therapy containing a combination of two antibiotics as (amoxicillin, clarithromycin, tinidazole, or metronidazole) and one proton pump inhibitor as (omeprazole, lansoprazole, pantoprazole, or rabeprazole). However, with the relatively recent emergence of clarithromycin resistant strains of H. pylori, the efficacy of the standard triple therapy has reportedly decreased to <80% [10].This resistance is caused by point mutations within the peptidyltransferase-encoding region of the 23S rRNA,in which an adenine residue is replaced by a guanine or a cytosine residue in different positions: A2142C, A2142G, and A2143G [11].These mutations decrease the binding of clarithromycin and all other macrolides to ribosomes leading to class-wide resistance [12]Phenotypic methods as agar diffusion for the E-test have been mainly used for routine detection of clarithromycin resistance However, these methods are time consuming [13].Meanwhile ,several PCR-based techniques have been developed to detect these forms of mutations, such as PCR-restriction fragment length polymorphism (RFLP) [14], PCR-DNA-enzyme immunoassay and reverse hybridization line probe assay. Real-time PCR methods are based on amplification of a fragment of the 23S rRNA gene of H. pylori followed by melting curve analysis [15].

The implementation of Real-time PCR into the clinical laboratory will allow both the identification of H. pylori and the assessment of clarithromycin resistance in paraffin- embedded gastric biopsy specimens in less than 4hours.This technique can replace the time consuming culture and antimicrobial susceptibility testing [16].

The aim of our study was to use Real time PCR method directly on paraffin-embedded gastric biopsies from gastric carcinoma patients as a rapid and reliable single-step method for detection of clarithromycin resistance-associated gene mutations in H. pylori and to investigate the degree of association of H. pylori resistant strains with different types of gastric carcinoma.

MATERIALS AND METHODS

Twenty, paraffin embedded gastric biopsy specimens were obtained from the archives of Pathology department Faculty of Medicine Ain Shams University during the period 2014-2015.

Selection criteria include:

- Gastric biopsy specimens positive for gastric carcinoma (adenocarcinoma: diffuse or intestinal type, or MALT lymphoma,)

-Association with H. pylori infection (confirmed by Giemsa stain).

Patient's data including age, sex, and endoscopic features for presence or absence of gastric erosions were collected.

Light microscopic evaluation of gastric biopsy specimens:
The cases were reevaluated for: Site of biopsy (fundus, body, pyloric antrum)

Type of malignancy (adenocarcinoma: diffuse or intestinal type, or MALT lymphoma)

The histopathological variables (H. pylori density, neutrophil activity and mononuclear infiltration, gastric atrophy, intestinal metaplasia and dysplasia were graded on a scale of 3 (mild, moderate and severe)according to the updated Sydney Classification system [17].

Detection of point mutations in the 23S r RNA gene of H. pylori by real-time PCR

A real-time PCR-hybridization assay was used directly on DNA obtained from paraffin-embedded gastric biopsies to detect point mutations conferring resistance to clarithromycin. First, a 267-bp fragment of (HPY-S) and (HPY-A).Amplification was detected using a 50 Light cycler red. The primers were analyzed for 3'-terminal specificity to assure that they were specific to H. pylori. The amplified products were detected using two probes:a) the sensor probe, which anneals with the mutant region ,b) the anchor probe, which annealswith three bases upstream from the former (GenBank accession number U27270).as shown in Table 1 and Table 2

Table 1: Showing primer names and it's sequence.

Primer name	Sequence	Amplicon size
HPY-S	5'-AGGTTAAGAGGATGCGTCAGTC-3'	1931/ 1952
HPY-A	5'-CGCATGATATTCCCATTAGCAGT-3'	2197 / 2175

Table 2: Showing the detection probes and it's sequence.

Detection probes	Sequence	Amplicon size
Sensor probe	5'-GGCAAGACGGAAAGACC-3'	2504 / 2520
Anchor probe	5'-TGTAGTGGAGGTGAAAATTCCTCCTACCC-3'	2473 / 2501

By using the Light Cycler thermocycler (Roche Diagnostics, Neuilly sur Seine, France), the PCR and hybridization reactions were carried out in glass capillaries in a volume of 20 µl containing 3 µl of template DNA, 1.6 µl of MgCl2 (25 mM),0.4 µl of forward and reverse primers (20 µM each), 0.2 µl of sensor and anchor probes (20 µM each), and 2 µl of Fast start DNA Master Hybridization Probes(Roche Diagnostics). PCR amplification comprised an initial denaturation cycle at 95°C for 10 min, followed by 50 amplification cycles (with a temperature transition rate of 20°C/s) consisting of 95°C for 0 s, annealing at 60°C for 10 s, and extension at 72°C for 17 s. After amplification a melting step was performed, consisting of 95°C for 0 s, cooling to 45°C for 30 s (with a temperature transition rate of 20°C/s), and finally a slow rise in the temperature to 85°C at a rate of0.1°C/s with continuous acquisition of fluorescence decline. DNA extracted from the known samples was used in each run as a positive control. Melting curve analysis of DNA from the cultured reference strains produced three melt curves, with Tm of B61.2, 51.8, and 52.2 1C for the wild-type strain and mutant strains A2142G and A2143G, respectively [14].We used the difference in melting curves to distinguish between the wild-type strain and mutant strains.

Statistical Analysis

Data were collected, revised, coded and entered to the Statistical Package for Social Science (IBM SPSS) version 20. Qualitative data were presented as number and percentages while quantitative data were presented as mean, standard deviations and ranges. The comparison between two groups with qualitative data were done by using Chi-square test and/or Fisher exact test was used instead of Chi-square test when the

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expected count in any cell was found less than 5. The comparison between two groups regarding quantitative data with parametric distribution was done by using Independent t-test. The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the p-value was considered significant at the level of < 0.05, and highly significant at the level of P < 0.001.

RESULTS

Our results done on twenty, paraffin embedded gastric biopsy specimens showed that the mean age for the patients were 53.60 ± 10.03. Twelve samples were obtained from male and 8 from females. Regarding the type of gastric malignancy, diffuse gastric carcinoma "signet ring" was found in 15 biopsies (75%), intestinal type adenocarcinoma (G2) in 3 biopsies (15%), and MALT lymphoma in 2 biopsies (10%)as shown in Table (3). Moderate H pylori infection was found in 12 cases (60%), while severe H pylori infection was found in 8 cases (40%)(figure 1).No cases had gastric atrophy or intestinal metaplasia, while fourteen cases had dysplasia (7 were mild dysplasia (35%) and 7 were moderate dysplasia (35%)(figure 2).All the 20 paraffin embedded gastric biopsy specimens showed PCR positivity for H. pylori; the mutated strains associated with resistance (A2142G orA2143G) were found in 12 specimens(60%) (figures 3),while the wild nonresistant strains were found in 8 specimens (40%).

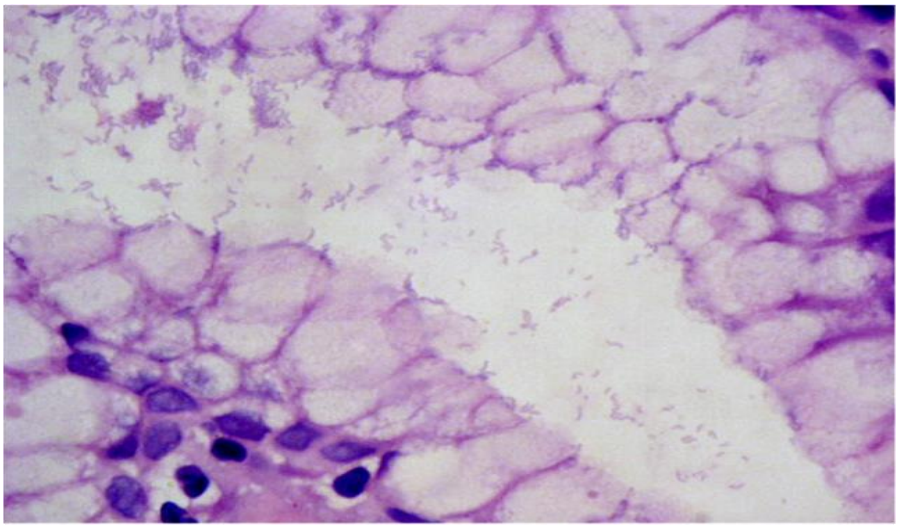

Figure 1: Gastric antral biopsy showing severe H pylori infection in gastric pits (H&E x1000, oil immersion)

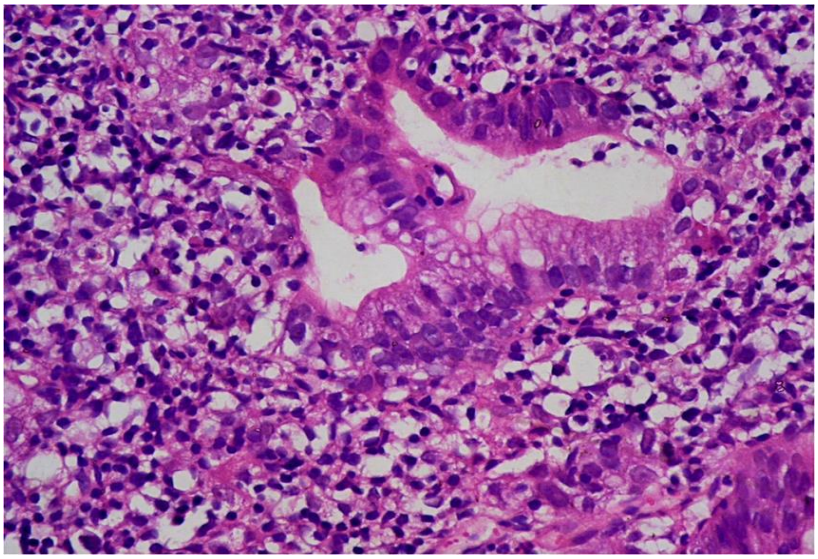

Figure (2): Gastric biopsy with evidence of H. pylori infection in gastric pits showing moderate dysplasia with hyper chromatic stratified nuclei, focal loss of polarity, and back to back arrangement of the glands (H&E x400).

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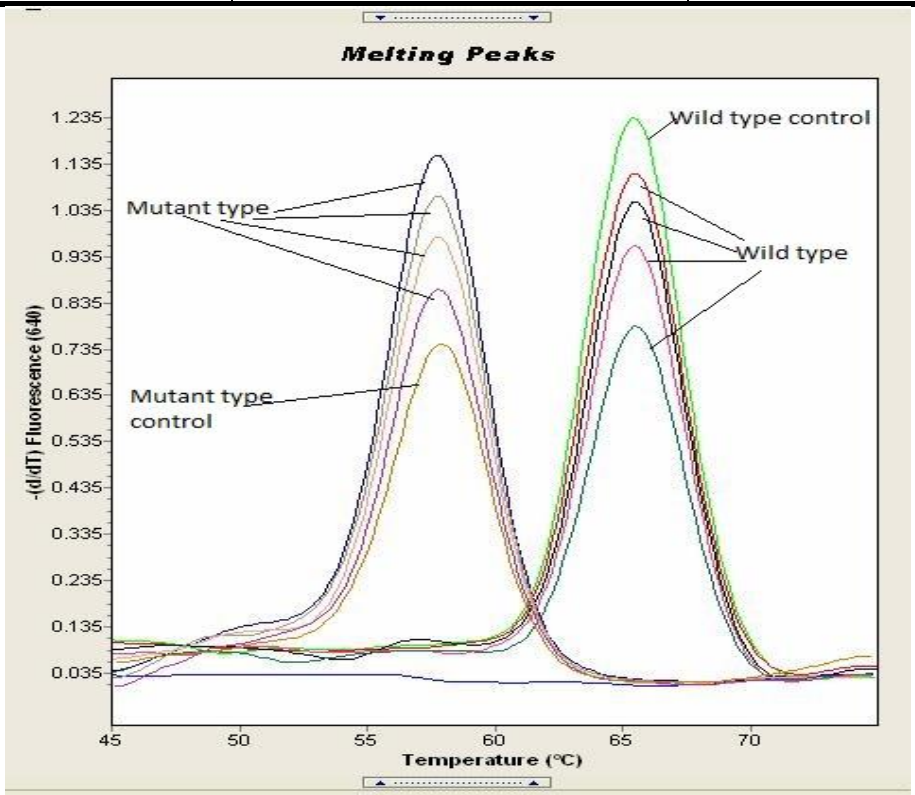
Table (3): Gastric carcinoma characterization.

		No.= 20
Site	Antrum	15 (75%)
	Body	2 (10%)
	Body& antrum	3 (15%)
Erosion	Absent	12 (60%)
	Present	8 (40%)
Hpylori	Mild	0 (0%)
	Moderate	12 (60%)
	Severe	8 (40%)
Chronicity	Mild	0 (0%)
	Moderate	17 (85%)
	Severe	3 (15%)
Activity	Mild	0 (0%)
	Moderate	11 (55%)
	Severe	9 (45%)
Gastric atrophy	Absent	20 (100%)
	Present	0 (0%)
Intestinal metaplasia	Absent	20 (100%)
	Present	0 (0%)
Dysplasia	Absent	6 (30%)
	Mild	7 (35%)
	Moderate	7 (35%)
	Severe	0 (0%)
Type of malignancy	Diffuse gastric carcinoma "signet ring"	15 (75%)
	Intestinal type adenocarcinoma (G2)	3 (15%)
	MALT lymphoma	2 (10%)
PCR	Non resistant strains	8 (40%)
	Resistant strains	12 (60%)



Figure(3): Melting peaks of both mutant and wild strains.

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There was a significant correlation (p value=0.009) between the presence of resistant H.pylori strains and gastric erosions (10 out of 12 cases or 83.3%), while no significant correlation was found with all the other parameters of Sydney classification system (table 4).

Table (4): Correlation between all parameters and and presence or absence of resistant H.pylori:

		Non resistant strains	Resistant strains	Independent t-test	
		No.= 8	No.= 12	t/X^2*	P-value
Age	Mean ± SD	54.50 ± 5.15	53.00 ± 12.48	0.035	0.852
	Range	43 - 59	35 - 74		
Sex	Female	3 (37.5%)	5 (41.7%)	0.320*	0.753
	Male	5 (62.5%)	7 (58.3%)		
Site	Antrum	6 (75.0%)	9 (75.0%)	2.222*	0.329
	Body	0 (0.0%)	2 (16.7%)		
	Body& antrum	2 (25.0%)	1 (8.3%)		
Erosion	Absent	6 (75.0%)	2 (16.7%)	6.806*	0.009
	Present	2 (25.0%)	10 (83.3%)		
Hpylori	Mild	0 (0.0%)	0 (0.0%)	1.250*	0.264
	Moderate	6 (75.0%)	6 (50.0%)		
	Severe	2 (25.0%)	6 (50.0%)		
Chronicity	Mild	0 (0.0%)	0 (0.0%)	2.353*	0.125
	Moderate	8 (100.0%)	9 (75.0%)		
	Severe	0 (0.0%)	3 (25.0%)		
Activity	Mild	0 (0.0%)	0 (0.0%)	2.155*	0.142
	Moderate	6 (75.0%)	5 (41.7%)		
	Severe	2 (25.0%)	7 (58.3%)		
Gastric atrophy	Absent	8 (100.0%)	12 (100.0%)	NA	NA
	Present	0 (0.0%)	0 (0.0%)		
Intestinal metaplasia	Absent	8 (100.0%)	12 (100.0%)	NA	NA
	Present	0 (0.0%)	0 (0.0%)		
Dysplasia	Absent	2 (25.0%)	4 (33.3%)	4.921*	0.085
	Mild	5 (62.5%)	2 (16.7%)		
	Moderate	1 (12.5%)	6 (50.0%)		
	Severe	0 (0.0%)	0 (0.0%)		
Type of malignancy	Diffuse gastric carcinoma	8 (100%)	7 (58.3%)	4.444*	0.108
	Intestinal type adenocarcinoma (G2)	0 (0.0%)	3 (25.0%)		
	MALT lymphoma	0 (0.0%)	2 (16.7%)		

⋆Chi-square test.
NA: NotApplicable.

DISCUSSION

Gastric cancer represents the fourth most common cancer and the second most common cause of malignancy-related death worldwide [18,19]. Infection with H. pylori is the strongest known risk factor for gastric cancer [20]. About 89% of the global gastric cancer burden and 5.5% of all malignancies worldwide are attributable to H. pylori-induced inflammation and injury [21].

Gastric inflammation induced by chronic H. pylori infection increases the risk of progression to adenocarcinoma through steps of gastric transformation, including atrophic gastritis, intestinal metaplasia, and dysplasia[22].The emergence of antimicrobial resistance in H. pylori represents a serious public health challenge because of the high prevalence of infection and high incidence of severe sequelae [23].Unfortunately, primary clarithromycin resistance, due to point mutations in the peptidyltransferase loop of the 23S r RNA , is increasing worldwide, and it has been regarded as a main factor for H. pylori eradication therapy failure [24].

In this study, we attempted to use Real time PCR method directly on paraffin-embedded gastric biopsies from gastric carcinoma patients as a rapid and reliable single-step method for detection of clarithromycin resistance-associated gene mutations in H. pylori, and to investigate the degree of association of H. pylori resistant strains with different types of gastric carcinoma. All the 20 paraffin- embedded gastric biopsy specimens in our study showed PCR positivity for H. pylori; the mutated strains associated with resistance (A2142G or A2143G) were found in 12 specimens (60%) ,while the wild non resistant strains were found in 8 specimens (40%).

We detected a statistically significant correlation between H.pylori resistant strains and gastric erosions. This result wasn't in agreement with Duck et al.,(2004) who found no significant correlation between H.pylori resistance and any abnormal endoscopic finding [25].Moreover, we found no statistically significant correlation between H. pylori resistant strains and all other parameters of Sydney system for scoring of gastritis.However,Gazi et al., (2013)found a statistically significant association between almost all Sydney classification parameters and genetic alterations in 23S rRNA of H. pylori[26]. This difference may be due to limited number of cases which was an important limitation in our study.

Although there was no statistical significant correlation between H.pylori resistant strains and histologic type of gastric tumor in our study, yet it was observed that all cases of MALT lymphoma and intestinal type gastric adenocarcinoma (non- signet ring carcinoma) were positive for resistant strains, while all non-resistant strains were found among cases of diffuse type (signet ring) gastric carcinoma (SRC).While non-signet ring carcinoma is often multifactorial, H. pylori induced chronic gastritis is involved in most cases. Moreover, H. pylori infection of the stomach is considered a major risk factor for gastric MALT lymphoma [27], and approximately 90% of patients with gastric MALT lymphoma are persistently infected with H. pylori [28].This may partially explain why all cases of intestinal type gastric carcinoma (3 out of 3) and MALT lymphoma(2 out of 2) in our series were positive for H.pylori resistant strains. On the other hand, the role of H. pylori in signet ring type is still more controversial. Indeed, since wide eradication of H. pylori, an H. pylori negative gastric cancer (H. pylori NGC) entity has been emerging. This entity may include several subtypes, such as gastric adenocarcinoma of the fundic gland (GA-FG-CCP) and SRCC, thus questioning the role of H. pylori in these histologic subtypes [29].Moreover, since the advent of treatment to eradicate H. pylori, the incidence of gastric adenocarcinoma has decreased, while the incidence of signet ring cell carcinoma is rising as it was found in 8% to 30% of gastric cancers [30]. In the present study, H. pylori resistant strains were found in 7 cases of diffuse gastric carcinoma while non resistant strains were detected in 8 cases. We didn't find in the literature any previous studies concerning the prevalence of H. pylori resistant strains in signet ring cell carcinoma.

Eradication of H. pylori infection is associated with regression of gastric MALT lymphoma in the large majority of patients. In a systematic review of the data from 32 published studies that included 1408 patients with gastric MALT lymphoma, the complete histological response rate of H. pylori eradication was 78% [31].Meanwhile, this eradication has been reported to be beneficial even in patients without laboratory confirmation of H. pylori infection [32,33].That's why failure of treatment due to presence of resistant strains may implicate a risk factor for persistence of MALT lymphoma.

The real-time PCR method used in this study permits the rapid detection of clarithromycin-resistant H. pylori directly from paraffin -embedded gastric biopsy specimens in cases where cultures are not routinely performed, or where unsuspected H. pylori -associated gastritis has been detected on histopathological examination. This could potentially have a major impact on clinical management of H. pylori-associated gastritis and carcinoma, allowing for the timely assessment of clarithromycin resistance status leading to a decrease in treatment failure rates.

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