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Evaluation of Clarithromycin and Metronidazole Resistance of Helicobacter Pylori Infection in Symptomatic Iranian Children

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Abstract

Background

Helicobacter Pylori (H. pylori) as a gram-negative bacterium is the most common infection of the gastrointestinal tract, and worldwide it affects the children over three years of age. H. pylori could cause gastrointestinal and extra-intestinal manifestations. Antibiotic resistance can happen primarily and occurs during treatment. We aimed to evaluate the resistance gene of H. pylori obtained from gastric biopsy by polymerase chain reaction (PCR) method in Iranian children over 3 years old.

Materials and Methods

This study was a cross-sectional to evaluate the resistance gene of H. pylori obtained from gastric biopsy by polymerase chain reaction method for metronidazole and clarithromycin in children over three years old referring to the Mofid Children's Medical Center in Tehran, Iran.

Results: Finally, data from seventy-nine samples included (mean age=10.7 years and male gender = 60.8%). *Beta Globulin (BG)* gene were detectable in 75 (94.93%) specimens of 79 (100%). Seventeen out of 75 specimens showed positive results for molecular detection of H. pylori. The results of RFLP-PCR technique showed that mutation of *RdxA* gene in seven of 17 (41.1%) for Metronidazole resistance and one case of 17 (5.8%) mutation of 23Y RNA gene that leads to clarithromycin resistance.

Conclusion

Regarding the results of our study, it is better to check microbial resistance by culture and antibiogram for the antibiotic regimen of the first and second line of H. pylori treatment in children.

Key Words: Antibiotic Resistance, Children, Helicobacter Pylori, Infection.

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1- INTRODUCTION

Helicobacter Pylori (H. pylori) is a gram-negative non-spore-forming bacterium which grows under microaerophilic conditions at an optimum temperature of 35-37°C and high humidity (1, 2). It is one of the most common infections of the Gastrointestinal tract that affects most children over three years old (3). The prevalence of H. pylori is different according to ethnic group, geographic area, healthy condition, family size and cultural habits (4). The prevalence of H. pylori is low in westernized and developed countries (less than 10%) with proper healthy condition, on the other hand in developing countries associated with high rate (about 30% up to 80%) (3). Studies from the Netherlands, Turkey, China, Tunisia and rural Alaska have reported prevalence rates of H. pylori in children 1.2%, 30.9%, 13.1%, 51.4%, 86%, respectively (5-8).

H. pylori can cause gastrointestinal manifestations such as gastritis ulcer disease (gastric ulcer and duodenal ulcer), gastric carcinoma, gastrointestinal bleeding, Mucosa-Associated Lymphoid Tissue Lymphoma (MALT), and extra-intestinal manifestations such as iron deficiency anemia, Failure to thrive and micronutrient deficiency, chronic idiopathic thrombocytopenia, and short stature (9-14). Gastritis is an inflammation or injury of gastric mucosa and epithelium by an autoimmune response and hypersensitivity reactions that is usually caused by H. pylori infection (15). The perfect test for diagnosis of H. pylori should be highly accurate, noninvasive, inexpensive and readily available and capable of discriminating between active HP infection and past infection (2). H. pylori diagnostic tests can classify into two types of invasive (culture, histopathology, Rapid Urease Test [RUT]), and non-invasive (serologic test ،Urea Breath Test (UBT), stool antigen test). Invasive tests are more sensitive than non-invasive (16). If H. pylori is detected even in asymptomatic children, the eradication protocol is recommended (17). H. pylori treatment mostly is a combination therapy of three or four drugs regimen including Amoxicillin, Clarithromycin, Tetracycline, Metronidazole plus PPIs with or without bismuth compounds. H. pylori eradication regimen should have with high cure rates approximately 80%, minimal bacterial resistance and without significant side effects. Proton pump inhibitors, combined with antibiotics, prevent antibiotic degradation in the acidic pH of the stomach and increase the bactericidal effect. Since proton pump inhibitors and H2 blockers are not able to reduce the pH to 7, antibiotics used for H. pylori treatment need to be able to work in minimally acidic environments (18). However, complications and antibiotic resistance sometimes fail the treatment (19). Treatment failure can attribute to poor patient compliance, inadequate drugs intake (in dose or time), antibiotic resistance, and recurrence (18). Evidence shows that antibiotic resistance attributed to chromosomal mutations. In 20% of cases, H. pylori infection treatment associated with antimicrobial resistance (20). Although the gold standard diagnostic test for H. pylori is a culture with an antibiogram, because of the implementation problems in the culture and performing standard tests for determining the sensitivity to H. pylori, there is little information about the antibiotic resistance of these bacteria in childhood in Iran. Therefore, the aim of this study was to evaluate the resistance gene of H. pylori obtained from gastric biopsy by polymerase chain reaction (PCR) method in children over 3 years old referring to the Mofid Children's Hospital of Tehran, Iran, regarding two conventional antibiotics to help the election of antibiotics with low resistance for treatment of gastritis.

2- MATERIALS AND METHODS

2-1. Study design and setting

This study is a cross-sectional for determining the antibiotic susceptibility pattern of Helicobacter strains to antibiotics of metronidazole and clarithromycin in children older than three years old. That conducted after obtaining approval from the ethics committee of Shahid Beheshti University of Medical Sciences, obtaining necessary permits (325/12 Oct 2014), and providing the sampling permit to the Mofid Children's Hospital, Tehran, Iran.

2-2. Patients

A pediatric gastroenterologist performed a history taking and clinical examination. The inclusion criteria were patients with a possible diagnosis of H. pylori gastritis (UBT positive and stool antigen positive), and positive finding in the history and physical examination for Helicobacter gastritis between ages 3–18 years old that enrolled for endoscopy. The exclusion criteria were patients under three years or older than 18 years of age and functional abdominal pain. Then the objectives of the study were presented to their parents and if they consented to participate in the study. Informed consent obtained for endoscopy. The sample size was 87 children according to 55% prevalence resistant of metronidazole and alpha 5% (21). According to the endoscopic view of the stomach (including nodularity, gastric and duodenal ulcers, rugal hypertrophy), three samples of an antral biopsy taken for histopathological study , rapid urea's Test (RUT), PCR study and molecular identification in a transport media transferred to Pediatric Infectious Disease Research Center laboratory. If their RUT was negative or transfer conditions are inappropriate or more than 2 hours, the samples excluded from the study. Totally eight samples excluded from the study due to contamination or insufficient volume. The DNA extractions of all 79 samples have been extracting by specific extraction kit (QIAamp® DNA Mini Kit. Cat. No. 51304) according to manufacturer's instruction. The qualities of all extraction have been examined by amplification of beta globulin (BG) gene by conventional PCR (**Table.1**) (22).

Table-1: Primers used for amplification.

Primer name	5'-3' sequence	Reference
glmM-F	AAGCTTTTAGGGGTGTTAGGGGTTT	(23)
glmM-R	AAGCTTACTTTCTAACACTAACGC	
BG-F	CAACTTCATCCACGTTCACC	(24)
BG-R	ACACAACTGTGTTCACTAGC	
rdxA-F	AATTTGAGCATGGGGCAGA	(25)
rdxA-R	GAAACGCTTGAAAACACCCCT	
Cla18	AGTCGGGACCTAAGGCGAG	(26)
Cla3	AGGTCCACCACGGGGTCTTG	

2-3. Measurement

Molecular detection of H. pylori has done by the proliferation of glmM gene as a specific gene to molecular identification of HP on all specimens with BG PCR positive results. Metronidazole resistance has surveyed by detection of deletion on RdxA gene by PCR. The expected the gene was wild if 850bp but the gene was mutated if PCR product was 650bp (22). All primers shown in **Table.1**. PCR mixture includes; 12 µl PCR master mix (Ampliqon, Korea), 10µl sterile deionized water, two µl template DNA and 0.5 µl of each primer in total volume 25µl. PCR conditions were carried out according to initial denaturation at 94 ºC for 5 minutes followed by 30 cycles of 94 ºC for one minute, annealing for 1 minutes at 50°C,

an extension for 1 minutes at 72 ºC and final extended for 7 minutes at 72 ºC (25). 3'-mismatch PCR was used to detect A2142C point mutation in an internal region of 23s rRNA gene that causes resistance to Clarithromycin (26) with the primers in **Table.1**. In this case, there was no fragment, and none of the PCR product observed if the gene was the wild-type. While a 700bp fragment produced if the A2142C point mutation took place. 3'-mismatch PCR condition was as follow: reactions were carried out in 25µl mixtures containing12 µl PCR master mix (Ampliqon, Korea), ten µl sterile deionized water, one µl template DNA and one µl of each primer. Initial denaturation at 94°C for five minutes followed by 30 cycles of denaturation at 94°C for one min, annealing for one min at 55°C, extension at 72°C for one minute. The final extension step extended to five min at 72°C (27).

2-4. Outcome and Statistical analyzing

Diagnosis of gastritis and grading of chronicity and activity based on Sydney system grading, which has no defined criteria and is subjective (28). Esophagitis defined as inflammation of the esophagus. Diagnosis of esophagitis and grading of chronicity and activity is subjective and based on the number of inflammatory cells, increased intraepithelial squiggle cells, papillary elongation, hydropic changes and spongiosis (29). The data were entered into the Microsoft Excel software and analyzed using the SPSS software (IBM SPSS Statistics 21.0 software). Considering descriptive analysis, mean and standard deviation (SD) used for quantitative variables and absolute and relative frequencies used for nominal and ordinal ones. P-value less than 0.05 were statistically significant.

3- RESULTS

During the preliminary study, 87 patients who had a positive UBT test or stool antigen entered the study, but 8 sample excluded from the study due to contamination or insufficient volume. Therefore, the total sample size was seventy-nine. Forty-eight cases (60.8%) were boys, and 31 cases (39.2%) were girls. The minimum, maximum and mean of age were 4, 18 and 10.7 years, respectively (**Table.2**). Eight (10.1%) had a previous history of H. pylori infection, and 31 cases (39.2%) had a positive family history of H. pylori infection in the first-degree relatives. In this study clinical presentation (signs and symptoms) were, nausea (96.7%), epigastric pain (96.7%), regurgitation (82.3%) (**Table.3**). All of the patients were RUT positive (100%) during endoscopy. The histopathological result described in the **Figure.1**. *BG* gene has been detectable in 75 specimens (94.93%) of 79. Seventeen (22.66%) out of 75 specimens showed positive results for molecular detection of H. pylori and *glmM* specific band had observed after gel electrophoresis of PCR product. The results of PCR showed that mutation of *RdxA* gene in seven of 17 (41.1%) for Metronidazole and mutation of 23s rRNA gene in one case of 17 (5.8%) for Clarithromycin (**Figure.2**).

Table-2: Relative Frequency of the Demographic feature in the study group

Demographic status	Number
Total	79(100%)
Male	48(60.8%)
Female	31(39.2%)
Previous history of H.P infection	8(10.1%)
Positive family history of H.P infection	31(39.2%)
Mean of Age	7.10 years

Table-3: Relative Frequency of the signs and symptoms finding in the study group

Symptom	Number (%)	Symptom	Number (%)	Symptom	Number (%)
Epigastric pain	74(93.7%)	Anorexia	21(26.6%)	Bloating	0(0%)
Regurgitation	65(82.3%)	Polydipsia	22(27.8%)	Dysphagia	0(0%)
Nausea	76(96.2%)	Fullness	37(46.8%)	Distention	14(17.7%)
Vomiting	18(22.8%)	Pyrosis	16(20.3%)	Constipation	3(3.8%)
Heartburn	15(19%)	post prandial abdominal pain	16(20.3%)	Hoarseness	5(6.3%)
Prone position	59(74.7%)	Diarrhea	6(7.6%)	Cough	13(16.5%)
Rumination	34(43%)	Teething	0(0%)	Sign	Number (%)
Constipation	20(25.3%)	Globus sensation	0(0%)	Wheezing	2(2.5%)
Awake sleep	62(78.5%)	Headeache	10(12.7%)	Epigasteric tenderness	72(91.1%)
Bad sleep	57(72.2%)	Early satiety	9(11.4%)	Distention	14(17.7%)
Halitosis	53(67.1%)	Food impaction	6(7.6%)	Dental carries	65(82.3%)
				FTT	0(0%)

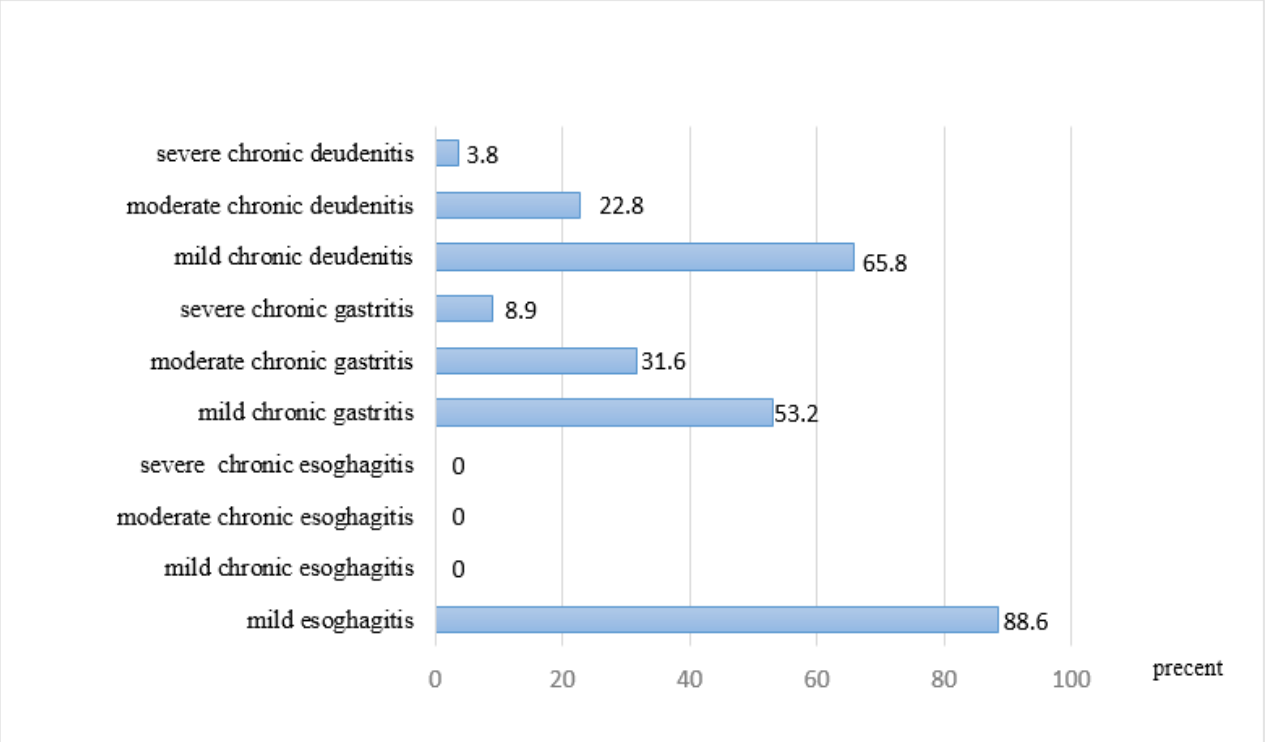

Fig.1: Relative Frequency of the different histopathology findings in the participants.

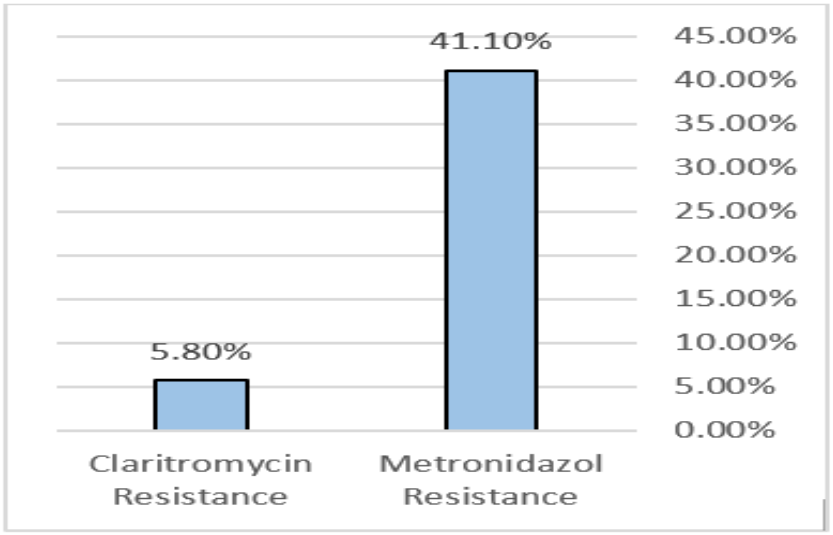

Fig.2: Relative frequency of the antibiotic resistance in the participants.

4- DISCUSSION

H. pylori, which infects almost half of the world's population, is a significant risk factor for chronic gastritis, gastric and duodenal ulcer and gastric cancer. Today, H. pylori eradication recommended as the most effective way to improve duodenal and stomach ulcers. One of the main reasons for H. pylori treatment failure is its antibiotics resistance. H. pylori infection occurs at a relatively high rate in early childhood in developing countries, and 70% to 90% of the population infected at the age of 20 years (14-17). A study by the Department of Pathology in Columbia reported that the prevalence of H. pylori infection was about 50%, which increased to 88.7% and 84%, respectively during three decades, with histopathology and microbiological tests (30). Saberi-Firozi reported resistance to metronidazole was 60% in adult population in Iran (31). Siavashi et al. (32), and Fallahi et al. (33) reported resistance to metronidazole in H. pylori isolates by PCR method as 95% and 54.14%, respectively in adult and children population. Ranjbary et al. study revealed was 75.5% and 3.35%, resistance to metronidazole and clarithromycin respectively in adult patients referring to the endoscopy department of Shahid Beheshti hospital of Shiraz (34). In this study, RFLP-PCR technique was used for 23Y RNA gene to identify the gene mutation that leads to clarithromycin resistance. Tangtawi et al. tried to determine the prevalence of clarithromycin resistance in H. pylori treatment using PCR method in Northern Thailand and found that the clarithromycin resistance in patients with H. pylori and gastrointestinal symptoms was 76.2%. They concluded that the H. pylori had a high resistance to clarithromycin in northern Thailand. Thus they did not recommend clarithromycin as the first line of the eradication regime of H. pylori (35). Martin et al. January found that the most commonly used mutation in clarithromycin resistance was in the A 2147 G position in the S 23 gene (36). Eghbali et al. study was also consistent with the results of the present study. They determined the point mutation of A2143 G on 23Sr, RNA gene chain H. pylori isolated from biopsy samples using the PCR technique and found 5.6% of cases were clarithromycin resistant (37). It is advisable to know the epidemiology of antibiotic resistance to select an appropriate antibiotic in each area38. It is noteworthy, to use biopsy with culture and antibiogram to measure antibiotic resistance to determine the best antibiotic regimen.

5- CONCLUSION

The results of this study emphasize that despite the increasing resistance to antibiotics commonly used against H. pylori in children, regarding the results of our and other studies, it is better to check microbial resistance by culture and antibiogram for the antibiotic regimen of the first and second line of H. pylori treatment in children. Additionally, applying a multi-drug regimen for treatment and eradication of H. pylori is still recommended until the emergence of new antibiotics. Finally, it emphasized that the culture and antibiotic resistance pattern are necessary for determining drug resistance patterns of this bacterium in the different geographical area before the onset of treatment.

6- CONFLICT OF INTEREST: None.

7- ACKNOWLEDGMENT

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