Clarithromycin resistance and 23S rRNA gene point mutations of *Helicobacter pylori* infection in children

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This study aims to evaluate *Helicobacter pylori* with clarithromycin resistant genotypes in Manisa region, Turkey. Two hundred patients, who received diagnosis of *Helicobacter pylori* infection histopathologically, were included. The sex, age and endoscopy indications of the patients were recorded. Polymerase chain reaction method was applied to determine the clarithromycin resistance rate and resistance genotypes at the histologic sections prepared from gastric biopsies that had been embedded in paraffin after fixation by formalin. *Helicobacter pylori* resistance to clarithromycin was found in 19/200 (9.5%) patients. 10/19 (52.6%) of these clarithromycin-resistant patients had A2143G mutation and 9/19 (47.4%) had A2142G mutation. A2142C mutation on 23S rRNA gene was not detected for any of the patients. Clarithromycin can be used as a first step treatment in the eradication of *Helicobacter pylori* for the children in our region; if the treatment fails for some patients, clarithromycin resistance, especially A2143G and A2142G mutations should be considered.

Key words: Helicobacter pylori, clarithromycin, genotype, polymerase chain reaction, child.

Helicobacter pylori (H. pylori) infection plays an important role in the development of chronic active gastritis, gastric and duodenal ulcer for both children and adults[1,2]. Getting *H. pylori* at an early age is an important risk factor in development of gastric adenocarcinoma and mucosa-related lymphoid tissue (MALT) lymphoma at adult age[3]. Some important complications that may accompany *H. pylori* infections during childhood are iron deficiency anemia resistant to treatment, short stature, autoimmune diseases and idiopathic thrombocytopenic purpura. Prevalence of *H. pylori* infection is 70-90% in developing countries and 25-50% in developed countries[4]. In our country 30-56.6% of children get infected with *H. pylori* and detection and treatment of the disease during childhood is important for preventing complications[5]. In the recent years, the highest level of success in eradication of *H. pylori* has been 70%[6]. Clarithromycin has

an important place among many treatment protocols offered for eradication of *H. pylori*. However, the increase of strains resistant to clarithromycin decreases the success level of eradication treatment[7]. The numbers of studies about children are less than those about adults on this subject in the literature. The objective of this study is to determine the clarithromycin resistance ratio and genotypic clarithromycin resistance for children with *H. pylori* in the Manisa region.

Material and Methods

Patients

The study included 200 patients, retrospectively, aged between 4 and 18 years, who had consulted at Celal Bayar University Medical Faculty Pediatric Gastroenterology Department between May 2009-June 2014, and underwent gastrointestinal system (GIS) endoscopy. The patients included in the study were selected

from the patients that were *H. pylori* positive according to the histopathological results. The patients who had previously received *H. pylori* eradication treatment, and proton pump inhibitor (PPI) and antibiotics treatment within the 8 weeks prior to endoscopy were not included in the study. The study was carried out between September 2014- July 2015. The study was accepted by the local scientific ethics committee of Celal Bayar University Medical Faculty, Manisa, Turkey. Permission was obtained from the child's parents for the study and endoscopic procedure. The sex, age and endoscopy indications of the patients were recorded from the demographic data of the patients.

Assessment of routine Upper GIS endoscopy and pathology for Helicobacter pylori

Olympus GIF-Q150 gastrointestinal videoscope (Olympus Corporation, Tokyo, Japan) was used to perform an upper GIS endoscopy to the patients. Gastric biopsy specimens were taken from antrum (two), incisura angularis (one) and corpus (two). These specimens were fixed in Hollande's solution in bottles as soon as they were taken from the patient. Following the routine biopsy follow-up, the specimens were properly placed vertically on the mucosa surface, embedded in paraffin and sections were taken from 3 levels. The sections were stained with Hematoxylin-Eosin (H&E) for histopathological examination and also with Toluidin Blue for detection of *H. pylori*. In the microscopic assessment, the antrum and corpus biopsies were scored separately using visual scala in accordance with the "Updated Sydney System" evaluation criteria. Consequent to scoring chronic inflammatory infiltration in lamina propria, epithelial neutrophilic infiltration, atrophy, intestinal metaplasia and *H. pylori* colonization degrees were defined separately.

Preparing section at paraffin blocks

In the course of the study, following upper GIS endoscopy routine biopsy specimens were forwarded to pathology for histopathological assessment and formalin-fixed, paraffin-embedded antrum and corpus biopsies, preserved in the pathology department, of the 200 *H. pylori* positive patients were incorporated in the study. For detection of polymerase chain reaction (PCR), paraffinized sections of 10 micron thick were taken from blocks containing antrum and corpus biopsies of each case.

DNA isolation and detection of clarithromycin resistance

After deparaffinization of paraffinized sections, QIAamp® kit (Qiagen, Hilden, Germany) was used for DNA isolation. Three-point mutations (A2143G, A2142G, A2142C) of *H.pylori* 23S rRNA, responsible for clarithromycin resistance were detected by Geno Type® HelicoDR kit (Hain Lifescience, Nehren, Germany) using PCR method. The kit was used in accordance with the instructions of the producing company. The strips generating *H. pylori* and 23S band were evaluated in terms of resistance mutation. The samples were considered to be sensitive to clarithromycin when 23 SWT (wild type) band occurred but mutation band did not appear and resistant to clarithromycin when any of the mutation bands appeared but 23 SWT did not occur.

Statistical Analysis

The data was assessed using descriptive statistics (number, percentage distribution, mean, standard deviation), chi-square and Fisher's exact test. $p < 0.05$ was taken as significance level.

Table I. Clarithromycin Resistance and Endoscopy Indications

Clarithromycin resistance		No	Yes		
			Clarithromycin resistance mutation		
			A2143G	A2142G	A2142C
Endoscopy indications	Dyspepsia n/total (%)	141/158 (89.2)	10/158 (6.3)	7/158 (4.4)	-
	Abdominal pain n/total (%)	15/17 (88.2)	-	2/17 (11.8)	-

Results

One hundred twenty seven of the 200 patients were girls (63.5%) and 72 were boys (36.5%), their mean age was 13.24 ± 2.99 years. Upper GIS endoscopy indications were dyspepsia (79%), anemia (10%), abdominal pain (8.5%) and GIS bleeding (2.5%). *H. pylori* resistance to clarithromycin was found in 19/200 (9.5%) patients. 10/19 (52.6%) of these clarithromycin-resistant patients had A2143G mutation and 9/19 (47.4%) had A2142G mutation. A2142C mutation was not detected for any of the patients.

Clarithromycin resistance was observed for the dyspepsia group 17/158 (10.7%), abdominal pain group 2/17 (11.8%). Clarithromycin resistance was not seen in anemia and GIS bleeding groups (Table I). A meaningful correlation was not set between upper GIS endoscopy indications and detection of clarithromycin resistance (p=0.38). Gastritis was determined for 151/181 (83%) and peptic ulcer was determined for 22/181 (12%) patients who had no clarithromycin resistant mutation. All of the clarithromycin resistant patients had gastritis, only four of them had duodenal ulcers. But there was no statistically significant relationship between clarithromycin resistance mutation and the presence of peptic ulcer (p=0.28) or gastritis (p=0.08) (Table II).

Discussion

The standard treatment of *H. pylori* eradication usually includes a triple treatment, which is a combination of PPI, amoxicillin and clarithromycin or metronidazole[8]. The most important reason behind the failure of treatment is the inconsistent medication and the resistance that develops against antibiotics. For many kinds of treatment, clarithromycin plays a key role in eradication of *H. pylori*. The worldwide increase of clarithromycin resistance reduces the ratio of success in *H. pylori* treatment[9]. According to the Maastricht IV/Florance consensus report, 15-20% is accepted as the threshold value to determine the regions having high or low clarithromycin resistance[6]. For regions where clarithromycin resistance is over 15-20%, the triple treatment including PPI and clarithromycin is not suggested to be used without making clarithromycin sensitivity tests[6].

Studies on adults in many countries reveal that *H. pylori* clarithromycin resistance varies between 2.1% and 30%[7,10-13]. In Turkey, clarithromycin resistance ratio is reported to vary between 5.4%- 48.2 % by using different methods[14-16]. A study in Turkey in 2004 included 102 *H. pylori* positive children and clarithromycin resistance was detected for 18.2% of the patients and with the first step treatment involving clarithromycin, eradication was attained for 75.5% of those patients[17]. Another study carried out in Izmir, which is a neighboring city of Manisa, in 2012 included 44 children and clarithromycin resistance was found to be 25.7% and *H. pylori* eradication could be attained for 46.4% of the patients, who received standard treatment comprising clarithromycin[18]. In 2014, 89 children were included in another study from a different part of Turkey and clarithromycin resistance was found to be 23.5%[19]. Factors such as the use of different methods, different ratios in using antibiotics for regions and the experience of people applying the method can be effective in identifying different resistant ratios in studies[20]. While clarithromycin resistance ratio differs from region to region, it is a fact that the resistance ratio has increased throughout the years. The increase in clarithromycin resistance in our country and the world is connected to

Table II. Relation Between Clarithromycin Resistance Mutation and Duodenal Ulcers/Gastritis

Ulcer	Clarithromycin resistance mutation		p*
	(+) n/total (%)	(-) n/total (%)	
(+) n (%)	4/19 (21)	22/181 (12)	0.28
(-) n (%)	15/19 (79)	159/181 (88)	
Gastritis			
(+) n (%)	19/19(100)	151/181 (83)	0.08
(-) n (%)	0	30/181 (17)	

*Fisher's exact test

its frequent use particularly in respiratory tract infections[21,22]. In our country, the unnecessary and non-prescribed use of antibiotics can be an important problem and this can explain regional differences.

Clarithromycin is a bacteriostatic antibiotic, belonging to macrolide group and connecting to peptidyl transferase region of 23S ribosomal RNA (rRNA) molecule V domain. In the Western countries, three point mutation (A2143G, A2142G, A2142C) in the V domain of 23S rRNA is responsible for more than 90% of adult cases for clarithromycin resistance[23]. These three mutations, where adenine residue changes place with guanine at 2143 and 2142 positions or with cytosine (A2142C) at 2142 position, are localized at 23S rRNA's peptidyl transferase region[24]. As a result of these point mutations, clarithromycin cannot get connected to ribosomes and cannot be effective. Because of the technical difficulties in culture and antibiogram implementations of *H. pylori* and because it is a fastidious microorganism, practical, cheap and applicable methods are required[25]. Molecular diagnosis tests are alternative methods that are easily standardized and are faster than culture based tests[7]. PCR, one of the molecular diagnosis methods, shows clarithromycin resistance with quite a high sensitivity of 98%[26]. PCR is a successfully applicable method in showing point mutations that are the causes of clarithromycin resistance in formalin-fixed, paraffin-embedded gastric biopsies in the archive[27]. It is an advantage of this method to be able to determine the possible effect of clarithromycin resistance in the failure of treatment through the biopsy samples of previously applied treatments retrospectively. It is reported that concerning the formalin-fixed, paraffin-embedded gastric biopsies, the sensitivity of the PCR method can be enhanced through optimal control of time of fixation by formalin and proper choice of paraffin sections[28]. PCR method is particularly more effective in identifying *H.pylori* infection for cases having less number of bacteria[28].

In our study, mutation A2142C was not found in any patient, A2143G and A2142G mutations were found to be the most frequent. There are several other studies whose results are consistent with our study[20,28,29-31]. The eradication ratio was lower for patients detected to have A2143G mutation[29]. Other studies about the use of clarithromycin in the eradication treatment of *H. pylori* assert that the existence of A2143G mutation negatively effects the treatment process[27,30]. Especially for pediatric patients, A2143G mutation is responsible for the failure of treatment with a ratio of 50% and A2142G and A2142C mutations with a ratio of 20%[29].

In the frame of the study we have applied upper GIS endoscopy to the patients who suffered from dyspepsia, abdominal pain, anemia and GIS bleeding, and we have detected *H. pylori* positivity. Among these patients, clarithromycin resistance was mostly observed for the dyspepsia group, followed by the group having abdominal pain. There was no significant relationship between upper GIS indications and detection of clarithromycin resistance. The fact that clarithromycin resistance was low for the group of abdominal pain and that it was not detected for other patients who went through upper GIS endoscopy for other reasons may be related with the low number of patients in these groups. In a study from Turkey, the most frequent endoscopy indication was abdominal pain for 89.2 % of the patients, and 18.2% clarithromycin resistance was found[17]. *H. pylori* positive patients showed very different clinical symptoms and this was considered to be the reason for not being able to establish a relation between *H.pylori* positivity accompanied by clarithromycin resistance and endoscopy indications. In this study, there was no statistically significant relationship between clarithromycin resistance mutation and gastritis or peptic ulcer. In another study which included only dyspeptic patients, 21.7 % clarithromycin resistance was detected, but no correlation was found between gastritis, gastric ulcer, duodenal ulcer and gastric cancer and clarithromycin resistance[32].

In conclusion, the success of *H. pylori* eradication is important with regards to prevention of complications that may develop. Concerning *H. pylori* eradication, it would be an advantage to know the region's resistance to treatment and mutation type before starting clarithromycin. The tests presenting clarithromycin resistance are not applied routinely at each center. Therefore, the studies carried out are important for obtaining regional data and for determination of that region's treatment strategy. Conclusively, our study revealed that

clarithromycin resistance was detected to be low for gastric biopsy samples in Manisa region and therefore it can still be the first choice in *H. pylori* eradication treatment. If *H. pylori* eradication fails, clarithromycin resistance, A2143G and A2142G mutations should be considered primary.

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