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Original article

Detection of A2142G, A2142C and A2143G clarithromycin mutations in *Helicobacter pylori* in Alexandria University Pediatric Hospital

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ABSTRACT

Background: *Helicobacter pylori* (*H. pylori*) colonizes the stomach and affect almost 50% of the world's population. Clarithromycin is considered a cornerstone for *H. pylori* treatment. Emergence of clarithromycin resistance (CLR-R) has played a major role in failure of *H. pylori* eradication both in adults and children. Clarithromycin resistance is mostly due to mutations in 23S rRNA gene: A2142G, A2142C, and A2143G. The aim of the current study is to determine the prevalence of CLR-R among *H. pylori* infected children with prior clarithromycin treatment. **Materials and Methods**: Multiple endoscopic gastric biopsies were collected from 50 *H. pylori* infected children after cessation of clarithromycin-based treatment. Samples were subjected to histopathological examinations, rapid urease test (RUT) and simultaneous molecular detection of *H. pylori* infection as well as CLR-R by multiplex Real-Time polymerase chain reaction (PCR). **Results**: Histopathological examinations and RUT revealed *H. pylori* in 74% and 92% of samples respectively. Molecular detection of CLR-R showed that 62.5% positive *H. pylori* cases were not harboring any of the tested mutations, while 25% harbored 2143A-G single mutation. Double mutations (2142A-C and 2143A-G) were detected in only 4 cases. Statistical significant correlation existed between both RUT and PCR results as well as between histopathological findings and PCR test results. **Conclusions:** A combination of histopathogy, RUT and multiplex PCR procedures offers a real benefit in the simultaneous diagnosis of *H. pylori* infection along with clarithromycin resistance status. Other mechanisms of clarithromycin resistance need to be investigated to explain treatment failure in absence of the previously detected mutations.

Introduction

Helicobacter pylori (H. pylori) is a Gram-negative spiral bacteria which colonizes human gastrointestinal tract specially the stomach. Almost half of the world's population is infected by this bacteria. It is mainly responsible for development of gastric clinical outcomes as gastritis, peptic ulcer diseases and gastric cancer. Moreover, it also

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participates in the development of many other extra-gastrointestinal diseases as cardiovascular, neurological, hematological and respiratory diseases [1].

Diagnosis could be carried out through invasive or non-invasive methods. Invasive methods in which the collected gastric biopsies could be analyzed histologically, culturing the bacteria, using rapid urease test (RUT) or through using polymerase chain reaction (PCR). Non-invasive methods include serological testing, urea breath test (UBT) and stool antigen test (SAT) [2].

Various regimens were developed for *H. pylori* eradication including first line, second line and third line therapy, all based on clarithromycin which is considered a cornerstone for *H. pylori* treatment. Clarithromycin act by binding to the bacterial 23S rRNA of the 50S ribosomal subunit suppressing its activity and thus inhibits protein synthesis. Unfortunately, clarithromycin resistance (CLR-R) is increasing resulting in eradication failure of *H. pylori* in both adults and children [3].

The most common reported mutations responsible for about 90% of the CLR-R are A2143G, A2142G and A2142C. These mutations are responsible for conformational changes resulting in decreased binding of clarithromycin to its target site. Other *H. pylori* resistance mechanism against clarithromycin is the expression and activation of efflux pumps. *hefC*, *hefF*, *hefI*. These three pumps of the RND family are reported to be involved multidrug efflux including clarithromycin [4].

The aim of this study was to determine CLR-R by detection of A2143G, A2142G and A2142C mutations among children attending El-Shatby University Hospital, Alexandria, Egypt.

Patients and Methods

Over a period of 15 months, 50 *H. pylori* infected children were enrolled in this study. Inclusion criteria for the participated children were: age above one year till fifteen years old, confirmed *H. pylori* infection by *H. pylori* SAT, previous treatment with clarithromycin based regimen for at least two weeks, and failure of treatment as diagnosed by persistence of positive *H. pylori* SAT performed 3-4 weeks after cessation of treatment along with the persistence of symptoms. Children parents were carefully instructed to stop any antibiotics or proton pump inhibitors for 4 weeks and 2 weeks respectively before performing the endoscopy.

After obtaining an informed consent, multiple endoscopic gastric biopsies were collected mostly from the stomach's antrum. Specimens were then subjected to histopathological examinations, RUT and molecular detection of CLR-R using Real-Time polymerase chain reaction (RT PCR).

Histopathological examination

Two formalin fixed biopsies for each gastric specimen were sent to the pathology laboratory for assessment. Specimens were then routinely stained with hematoxylin and eosin (H&E) stain to detect the attachment of *H.pylori* to the surface epithelium. The assessment criteria for the histopathological findings were associated with the degree of inflammatory cell infiltration, atrophy, and intestinal metaplasia.

Rapid urease test (RUT)

One biopsy specimen was inserted in a tube containing a confirmatory test RUT. The RUT is a test that detects the presence of urease in the gastric mucosa thus helps in confirming the presence of *H. pylori* infection through urea hydrolysis.

Molecular detection of *H. pylori* and clarithromycin resistance

DNA was extracted from tissue biopsies using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. DNA amplification was carried out by STRATAGENE PCR using Bosphore *H. Pylori* Genotyping Mix v1 ANATOLIA GENEWORKS® according to manufacturer instructions. It employs 2 multiplex PCR reactions, and an algorism including internal positive control to detect H. pylori as well as 23s rRNA mutations responsible for CLR-R.

The thermal profile used was composed of an initial denaturation for activation the HotStarTaq DNA polymerase at 95℃ for 15 min, followed by 40 cycles of a two-step amplification (Denaturation at 97℃ for 30 sec and annealing and extension at 63℃ for 45 sec)

Results

In the present study most of participated children were in age group 7 - 12 years old (70 %), female children were more common (56%). Abdominal pain was the most common presenting symptom (50%) followed by hematemesis (28%). Erythema (100%) followed by nodularity in 78% were the most common endoscopic findings. Other endoscopic findings were punctate hemorrhage (24 %), and gastric erosions (16%).

Upon performing the RUT, most of the gastric biopsy specimens 37/50 (74%) were positive, while by histopathological examination, *H. pylori* were seen attached to gastric epithelium in 46 (92%) specimens.

Molecular detection of CLR-R using RT PCR showed that *H. pylori* DNA were not amplified in 18/50 (36%) (True negativity as internal control of the test was amplified). Out of 32/50 in which *H. pylori* was detected, 20/32 (62.5%) were not harboring any of the tested mutations representing the most common genotype (wild type), followed by A2143G single mutation in 8/32 (26%) cases. Double mutations (A2142C & A2143G) were detected in only 4/32 (12.5%) cases.

No statistical significant correlation was found between histopathological findings of *H. pylori* and RUT results (*p* value = 0.275) as *H. pylori* was seen attached to the gastric epithelium in 46/50 (92%) of gastric biopsies of which 35/46 (76%) were urease producers upon performing RUT, while 11/46 (24%) were non-urease producers. This was not statistically significant.

However statistical significant correlation was demonstrated between RUT results and PCR test results (p <0.001), as the majority of PCR *H. pylori* positive cases; 30/32 (93.75%) showed positive RUT results, while the majority of urease negative 11/18 (61.1%) were negative for *H. pylori* by PCR and this was statistically significant **(Table 1)**.

By correlating histopathological findings of *H. pylori* and PCR test results, statistical significance was found as *H. pylori* was seen attached to the gastric epithelium in the majority of gastric biopsy specimens of which 32/46 (69.6%) were PCR positive, in addition, all of the four negative histopathological gastric biopsy specimens were also negative by PCR test and this was statistically significant (p =0.013) **(Table 2)**.

Table 1. Association between RUT results and PCR test results for *H. pylori*.

PCR test results	**Rapid urease test**				χ^2	^{FE}p
	Positive		**Negative**			
	Number	**Percentage %**	**Number**	**Percentage %**		
PCR test positive (n= 32)	30	93.75%	2	6.25 %	18.021*	<0.001*
PCR test Negative (n=18)	7	38.9 %	11	61.1 %		

χ^2: **Chi square test** FE**: Fisher Exact**

p: p value for comparing between **urease test** and **PCR test.**

*: Statistically significant at $p \leq 0.05$

Table 2. Association between histopathological findings of *H. pylori* and PCR test results.

Histopathological results (presence of *H. pylori* attached to the epithelium)	**PCR test results**				χ^2	^{FE}p
	Positive		**Negative**			
	Number	**Percentage %**	**Number**	**Percentage %**		
esent (n=46)	32	69.6 %	14	30.4 %	7.729*	0.013*
Absent (n=4)	0	0 %	4	100 %		

χ^2: **Chi square test** FE**: Fisher Exact**

p: p value for comparing between **PCR test** and **histopathological test results.**

Discussion

Helicobacter pylori infection in children differs from adults with respect to epidemiology, host responses, disease manifestations, and the complication rate. Furthermore, treatment options are limited in this population and antibiotic resistance rates continue to increase [5].

Our study was designed to investigate CLR-R by detection of A2143G, A2142G and A2142C mutations among children who received

treatment with clarithromycin and are still complaining of gastric and abdominal symptoms.

In Egypt, several studies had investigated CLR-R in adults [6-9], however, there is lack of studies in pediatric age group [10,11]. In this study, children aged between one to fifteen years with mean age of 8.2 ± 3.1 years were included. The majority of children (70 %) ranged in the age group of 7-12 years. Female children represented the higher percentage (56%).

Clinical symptoms of *H. pylori* infections in children are poorly expressed and nonspecific due to their young age. Presenting symptoms include epigastric pain, vomiting, and hematemesis. In the present study, the most common complains of children were epigastric abdominal pain (50%) followed by hematemesis (28%), and vomiting (20%). These findings were reported in several other studies [12-14].

The most common endoscopic findings in the current study were gastric erythema, non-atrophic gastritis, punctate hemorrhage, as well as gastric erosions. These findings were also reported by other studies [15,16].

The presence of nodular gastritis in *H. pylori* pediatric patients is considered to be specific. Histologically they represent lymphoid follicles hyperplasia with germinal centers in the suitable lamina of the stomach. With *H. pylori* eradication treatment nodularity in the mucosa is reduced [17].

In our study, nodularity was seen in (78%) of the cases. An Algerian study conducted by **Moubri et al.** [18] reported almost the same percentage of nodularity seen in this study (76,68%). Moreover, a study conducted by **Hidaka et al**. [19] reported antral nodularity in 21/25 of the *H. pylori* positive cases (84%).

Rapid urease test is a cheap, simple and rapid test used for the indirect detection of *H. pylori* through detection of urease in the gastric mucosa infections with high sensitivity and specificity [20]. Histopathology is considered to be the gold standard method in *H. pylori* diagnosis [2]. Both histopathological test and RUT provide an excellent diagnostic accuracy but *H. pylori* detection is decreased in case of gastric atrophy or bleeding peptic ulcers [21].

In the present study urease production was detected in (74%) of gastric biopsies, and by histopathological examination, *H. pylori* was seen attached to the surface of the epithelium in (92%) of the obtained biopsies However no statistical significance was found between the results of both methods.

A possible explanation for histological method and RUT limitations could be the patchy distribution of *H. pylori*. Furthermore, the noncompliance of some participant for not using antibiotics and proton pump inhibitors two weeks prior to endoscopic examination could explain the false negative results of RUT. Moreover, false-negative results of RUT can develop when *H. pylori* is present in the coccoid form, which will cause reduction in urease activity.

By correlating the results of RUT to the results of PCR in the current study; strong concordance between the two tests was found as majority of PCR positive cases; (93.75%) showed positive RUT results. In addition, the majority of urease negative (61.1%) were negative for *H. pylori* by PCR. This association was statistically significant ($p < 0.001$).

The prevalence of primary and acquired CLR-R is increasing worldwide and is highly variable [22,23]. This could be one reason for failure of *H. pylori* treatment found in real life clinical practice.

The global variation of CLR-R is due to the difference in macrolide consumption [24]. Several studies indicated that clarithromycin resistant strains are acquired by children more than adults [25-27]. This could be explained by the fact that children are more frequently exposed to macrolides during respiratory tract infection treatment [28].

Since there are some limitations to culture-based antimicrobial susceptibility testing, the “gold standard” method, resistance to clarithromycin has then been hardly performed in routine clinical practice [29].

Many point mutations in 23S rRNA have been reported to induce CLR-R phenotype of *H. pylori.* Mutations A2142G, A2142C, and A2143G located in the peptidyl transferase loop of the 23S rRNA are the most common mutations [30]. These 3 mutations are responsible for more than 90% of CLR-R isolates [31].

In this study, a real-time PCR-based method was used for the detection of the transition A-G in position 2142 or 2143, A-C in position 2142. We found that wild type *H. pylori* strain was the most common genotype representing 62.5% of PCR positive cases, followed by 25% cases harboring

A2143G single mutation. Double mutation (A2142C and A2143G) was detected in only 12.5%.

In agreement to our findings, the CLR-R rate in *H. pylori* resistant strains isolated from children in China was 36.7% (29/79) with A2143G mutation being the most common 82.8% (24/29) [32]. Moreover, in another recent study carried out on children in Wroclaw Medical University found that CLR-R was 68% and A2143G was the most frequently detected mutation 61% (38/62) [33].

A similar CLR-R rate was observed in **Tamayoa et al**. study in Northen Spain. They stated that percentage of CLR-R was (33.3%) among 63 gastroduodenal biopsies obtained from children which was also in agreement with resistance rates of more than (30%) obtained in a European multicenter study [26].

A higher rate of CLR-R was also stated in another pediatric study. **Mitui et al.** [34] found that 19/38 (50%) of the specimens contained *H pylori* with mutations significant for CLR-R. The predominant type of mutation detected was the A2143G mutation with percentage reaching (89%), as for the A2142G mutation it was found in only (10.5%) of the cases.

In an Egyptian study [10] that was conducted on 34 children referred to Gastroenterology center and Mansoura University Children hospital, Egypt from December 2014 till August 2015. A higher percentage of CLR-R was reported (68.4 %) than that of the current study. The study pointed out that the common point mutations were A2143G and A2142G of CLR-R using PCR-restriction fragment length polymorphism (PCR-RFLP). The most common type of mutation was for A2143G (53.4%) followed by A2142G (35.7%). Only 3 isolates could not be identified to have any of the two types of the studied mutations.

The highest CLR-R rate was seen upon comparing the results of the present study with the results of a similar study carried out by Alarcón, Vega, Domingo, **Alarcón T et al.** [35] a significant difference in the percentage of CLR-R was demonstrated; 12 cases in the present study (37.5%) compared to (89 %) in their study. However both studies agreed that A2143G mutation was the most common detected mutations.

Another opposing CLR-R rate was stated in South America which presented a difference in the prevalence of CLR-R among countries [36]. A Colombian study in which biopsies from 133 children aged between 3 and 17 years of age were analyzed. Results of PCR-sequencing of the 23S rRNA gene showed that 92% exhibited a genotype susceptible to clarithromycin, whereas only 8% showed mutations associated with CLR-R, A2143G mutation was the most common (4 cases), followed by the A2142G mutation (1 case). Additional mutations or double mutations were not observed. This value was much lower than those reported for Mexico (21.6%) and Brazil (27%) for pediatric populations which is related to different macrolide exposure in each population.

A possible explanation of our negative PCR results is that DNA extraction was performed on only one biopsy specimen while more than two specimens were used for histopathological examination and RUT. Another suggested reason is that *H. pylori* colonization is not uniformly distributed across the gastric mucosa even within antrum area, and therefore biopsy specimens removed from different sites may give disconcordant results.

Other possible explanation that defines the negative PCR results of 18 cases -true negativity as the internal control was amplified- reported in our study might be related to *H. pylori* strains harboring mutations of low prevalence that are not detected by the kit used in this study such as A2115G, A2445G, T2117C, T2182C, T2289C, T2717C, T2276C G2141A, G2220A, G2224A, G2225A, G2287A, C2245T, C2611A, C2041T, C2083T, C2273T, C2191T, C2399T and C2622T

In conclusion, a combination of histopathogy, RUT and multiplex PCR procedures offers a real benefit in the simultaneous diagnosis of *H. pylori* infection along with clarithromycin resistance status. The overuse of macrolides in our country could be linked to clarithromycin resistance in *H. pylori* found in this study. More point mutations should be searched for in order to perform an accurate assessment of CLR-R in *H. pylori* using a PCR-based method.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The study was approved by the ethics committee of the Medical Research Institute – Alexandria University which is constituted and operating according to ICH GCP guidelines and

applicable local and institutional regulations and guidelines which govern IRB operation.

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