Int.J.Curr.Microbiol.App.Sci (2016) *5*(1): 165-173

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 1(2016) pp. 165-173**
Journal homepage: http://www.ijcmas.com

Original Research Article doi: http://dx.doi.org/10.20546/ijcmas.2016.501.014

Molecular Study of Primary Clarithromycin Resistant *Helicobacter pylori* strains from Egyptian Centre

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Keywords

Helicobacter pylori, Clarithromycin resistance, RFLP-PCR

Article Info

Accepted:
22 December 2015
Available Online:
10 January 2016

A B S T R A C T

Helicobacter pylori (*H.pylori*) is a common infection in Egypt. However, there are few data available about its antibiotics susceptibility. The aim of the present study was to detect to the presence of Primary clarithromycin resistance among *H.pylori* isolated from gastric biopsies from children and adults by the use of PCR-RFLP technique for detection of 23S rRNA gene mutations. The study included seventy four adults patients and thirty four children refereeing for Gastroenterology centre and Mansoura University Children hospital, Egypt from December 2014 till August 2015. Endoscopic examination was followed by gastric biopsies for specific culture of *Helicobacter pylori* (*H.pylori*) and antimicrobial susceptibility for isolated strains. Further molecular studies were performed for detection of clarithromycin resistance by PCR-RFLP for detection of 23S rRNA gene mutations. *H.pylori* was isolated from 68.8% of samples, figure 1. The isolates were with convergent rates from adults (62.2%)and children (68.4%). The isolated *H.pylori* had marked resistance for clarithromycin (46.2%) and amoxacillin (20.8%) with lower rates of resistance to tetracycline (15.4%) and metronidazole (12.8%). In comparison of antibiotics resistance among adults and children, there were insignificant difference between both groups regarding Clarithromycin (P=0.20) Amoxacillin (P=0.9), tetracyclin(P=0.08) and metronidazole (P=0.4). The remarkable finding is the significant increase in presence of mutations in isolates from adults compared to that from children (P=0.006). 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. We conclude from this study that clarithromycin resistance among *H.pylori* isolates is a common finding associated with specific point mutations of A2143G followed by A2142G. Resistance also to other antibiotics amoxicilline, tetracyline are common. Extended studies should be performed on large isolates from Egypt to elucidate the efficacy of first line triple therapy.

Introduction

Helicobacter pylori (*H.pylori*) is microaerophilic Gram negative spiral bacilli spiral bacilli which is motile by flagella. The shape and motility of *H.pylori* adapted it to colonize gastric mucosa of around 50% of population around the world. It is claimed to cause various diseases of gastrointestinal tract that range from simple dyspepsia, gastric and duodenal ulcers up to gastric cancer (1-4). The prevalence of *H.pylori* increases with age in developing countries being 30-50% in childhood up to 90% in adults. Bad sanitary conditions and low socioeconomic standards with overcrowding are risk factors for such infection (5-7).

Universal guidelines for *H.pylori* treatment include combined triple therapy with proton pumb inhibitors like combination of esomeprazole, amoxicillin and clarithromycin for 7 to 14 days. Second line drug combination therapy included the use of bismuth is as effective as triple therapy and is sometimes recommended as second-line therapy (treatment failure). Clarithromycin antibiotic is an effective antibiotic included in first and second line treatment of *H.pylori* (2) Clarithromycin is semisynthetic macrolide antibiotic with broad spectrum bacteriostatic activity toward many organisms including *H.pylori*. Its therapeutic use in treatment of *H.pylori* is also attributed to its secretion in gastric mucosa (8; 9).

Antibacterial activity of clarithromycin is attributed to its inhibitory effects on protein synthesis in bacteria by reversible binding to peptidyl transferase loop of V domain of 23S ribosomal RNA molecule resulting in translocation of aminoacyl transfer-RNA is effectively blocked resulting in the inhibition of protein synthesis (8; 10).

However, it has been reported that the increase in the incidence of clarithromycin resistance is a major cause of *H.pylori* treatment failure (11; 12). The resistance to clarithromycin is due to its wide use as treatment of common infections in the respiratory tract and in skin. Resistance to clarithromycin results from modification of the target site of its action.

Target site modifications are associated with two pathways the first is the point modification of encoding gene of peptidyl transferase enzyme and the other pathway is post-transcriptional methylation of 23S rRNA region (13-15).

The mechanism of clarithromycin resistance in *H. pylori* is mainly associated with the following point mutations in 23S rRNA gene, A2143G, A2142G and A2142C (16; 17).

Laboratory methods for detecting *H.pylori* resistance include phenotypic and genotypic methods. Phenotypic method mainly depends on primary culture of *H.pylori* from different clinical samples followed by agar dilution method, the use of strips impregnated with antimicrobial agent gradient (e.g. E-test, bioMérieux, France) and disc diffusion method. The major disadvantage of this technique is the prolonged time until the results are available and failure to detect resistance in 10% of cases (13). Genotypic methods appear more accurate alternative to phenotypic method. For molecular detection of clarithromycin resistance among *H. pylori* strains the method depends mostly on analysis of the point mutations in 23S rRNA gene. These techniques can be applied for both cultured isolates, biopsy samples and stool samples (18). The most important molecular technique used to detect point mutations are PCR followed by restriction fragment length

polymorphism (RFLP). Recent use of Real-Time PCR beside other methods like, PCR-DNA enzyme immunoassay, mismatched PCR, hybridization and sequencing techniques are also applied (19).

There are scarce data about clarithromycin resistance among *H. pylori* isolates from Egypt.

The aim of the present study was to detect to the presence of clarithromycin resistance among *H.pylori* isolated from gastric biopsies from children and adults by the use of PCR-RFLP technique for detection of 23S rRNA gene mutations.

Materials and Methods

The study included 74 adults patients and 34 children refereeing for Gastroenterology centre and Mansoura University Children hospital, Egypt from December 2014 till August 2015. The patients were complaining from upper gastrointestinal pain, hematemesis or/ and dyspepsia requiring gastroendoscopic examination. The study was approved by ethical committee of Mansoura Faculty of Medicine, Egypt. Approved written consent was obtained from each adult patient and from parents of children patients.

From each patient antral gatric biopsy was taken from the greater curvature about 2 cm from pylorus. The biopsy was transported to the laboratory in sterile container.

Each biopsy was homogenized and spread over Columbia blood agar supplied with 5% sheep blood (Oxoid Columbia agar base). Plates were incubated at 37^0C for 10 days in a microaerophilic atmosphere by use of gas packs (Campy Pak; Becton Dickinson). Identification of *H. pylori* was made by Gram staining of the colonies, lack of aerobic growth on blood agar plates, and testing for the presence of urease, oxidase and catalase (20**)**.

The susceptibilities of *H. pylori* strains to amoxicillin, metronidazole, clarithromycin and tetracycline were assessed by disc dif-fusion. Disc diffusion was performed on Columbia blood agar supplied with 5% sheep blood after inoculation of the colonies density was adjusted to 0.5 McFarland turbidity by insertion of each disc on the medium for 72 hours at microaerophilic condition.

Later on isolated colonies were kept frozen and stored at –80°C in Scheadler anaerobe broth (Oxoid, UK) supplemented with fetal bovine serum (Sigma-Aldrich, Germany) and glycerol (POCH, Pand). Further molecular studies were performed for detection of clritheomycin resistance by PCR-RFLP.

Bacterial Genomic DNA Extraction

DNA was extracted after thawing of frozen culture and subculture on Scheadler agar supplemented with 5% sheep blood. Colonies were suspended in 1 ml of sterile saline and centrifuged (12000 rpm/3 min). Total bacterial genomic DNA was purified using Qiagen DNA extraction kit according to the manufacturer's recommendations.

Molecular Determination of the Clarithromycin Resistance with PCR-RFLP

Determination of the common point mutations (A2143G, A2142G) causing resistance to clarithromycin in *H. pylori* strains was performed by PCR followed by RFLP analysis. PCR assay was conducted using primers and thermal profiles described earlier (Agudo *et al.*, 2011) (K1 –sense:

CCA CAG CGA TGT GGT CTC AG and K2 – antisense: CTC CAT AAG AGC CAA AGC CC). The reaction mixture of the final volume 25 µl contained: 2 µl of genomic DNA, 2 µl of each primer, 5 µl of Buffer, 1.5 µl of MgCl2 (25 mM), 0.5 µl of PCR Nucleotide Mix (10 mM each), 0.125 µl of GoTaq® DNA Polymerase (5 u/µl) and Nuclease-Free Water (Promega, USA). The products was further studied by RFLP to detect point mutations.

The RFLP assay was carried out with Eco31I (BsaI) enzyme (Thermo Scientific, USA) in order to detect A2143G mutation, while BbsI enzyme (Thermo Scientific, USA) was used to detect A2142G mutation. The study included 112 gastric biopsies samples from 74 adults patients and 38 children patients. *H.pylori* was isolated from 68.8% of samples, figure 1. The isolates were with convergent rates from adults (62.2%62.2%) and children (68.4%), table2.

The isolated *H.pylori* had marked resistance for clarithromycin (46.2%) and amoxacillin (20.8%) with lower rates of resistance to tetracycline (15.4%) and metronidazole (12.8%), figure 2.

In comparison of antibiotics resistance among adults and children, there were insignificant difference between both groups regarding Clarithromycin (P=0.20) Amoxacillin (P=0.9), Tetracyclin(P=0.08) and Metronidazole (P=0.4). The remarkable finding is the significant increase in presence of mutations in isolates from adults compared to that from children (P=0.006), table 3. 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, table 4. *H.pylori* is reported as world wide spread infection responsible for wide spectrum of gastrointestinal disorders. It is usually acquired in childhood especially in developing countries. In the present study *H.pylori* was isolated from 68.8% of gastric samples. The isolates were with convergent rates for adults (62.2%) and children (68.4%)

It has been reported that *H.pylori* infection in children from developing countries may be detected up to 70% while in developed countries it ranges from 10-15%. For adults in developed countries in Europe it ranges from 5-20% (14)

According to standard guidelines for diagnosis of upper abdominal pain ether in children or adults, upper gastrointestinal endoscopic examination is the recommended diagnostic tool. Treatment only is recommended for patients with peptic ulcers by the combination of proton-pump inhibitor and two or more antibiotics, for 7-14 days, depending on resistance rates of geographic areas (23).

Clarithromycin is the most potent antibiotic involved in the management of H. pylori infections; therefore study of resistance to clarithromycin is important(32). out of Eighty-seven studies from 2009 to 2014 on H. pylori antimicrobial resistance in the different countries only 5 studies were done in Africa (31) ; the reported rate in Europe were lowest in Norway (5.9%) and highest in Portugal (42.35%), in Asia lowest rates were in Malaysia (2.4%) and highest were reported in China and India (46.54% and 58.8% respectively (31). Antibiotic resistance of *H.pylori* especially to clarithromycin is an emerging threat that is responsible for the majority of therapeutic regimen failure. The resistance pattern of *H.pylori* varies according to the geographical regions. To our best of knowledge there is no data bout *H.pylori* resistance toward the first class antibiotics.

Table.1 Digestion Thermal Profiles

Enzyme	Thermal cycle conditions following PCR	bp length of the segment
A2142G BbsI	37°C (24 h)	332 bp and 93 bp (21)
A2143G Eco31I (BsaI)	37°C (30 min), 65°C (5 min)	304 bp and 101 bp (22)

Table.2 Positive Culture of *H.pylori* According to Age of Patients

	Adults (n==74)	Children (n=38)
Positive Culture	46(62.2%)	26(68.4%) P=0.08

Table.3 Comparison of Antibiotics Resistance and Presence of Gene Mutations According to Age

Antibiotics Resistance	Adults (n=46)	Children (n=38)	
Clarithromycin	28(60.8%)	8(30.8%)	P=0.2
Amoxacillin	22(47.8%)	10(38.55)	P=0.9
Tetracyclin	13(28.3%0	4((15.4%)	P=0.08
Metronidazole	10((21.7%)	5(19.2%)	P=0.4
Presence of mutations	22(47.8)	3(11.5%)	P=0.006

Table.4 Types of Points Mutation among *H. pylori* Isolates

	N0. %
Point mutations A2143G	15(53.4%0
Point mutations A2142G	10(35.7%)
Non identified	3 (10.7%0

Figure.1 Positive and Negative Culture of *H.pylori* among Patients

Figure.2 Antibiotics Resistance among *H.pylori* Isolated Strains

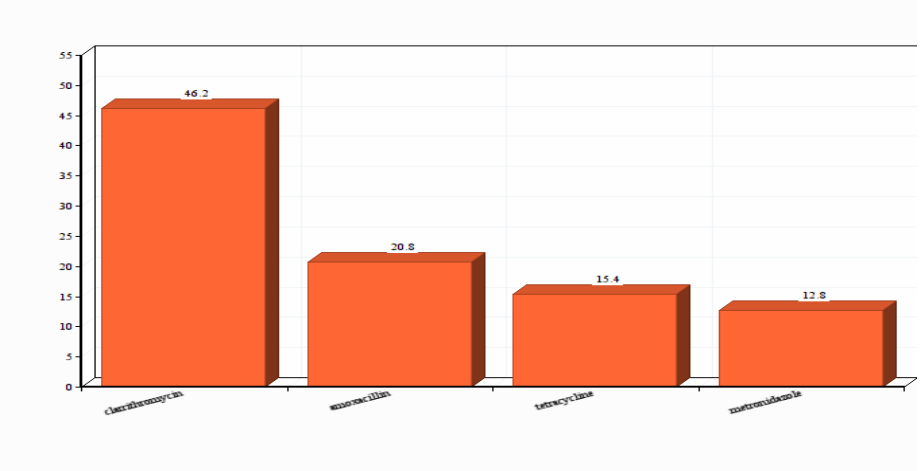

Figure.3 RFLP-PCR for *H.pylori* resistant and susceptible strains

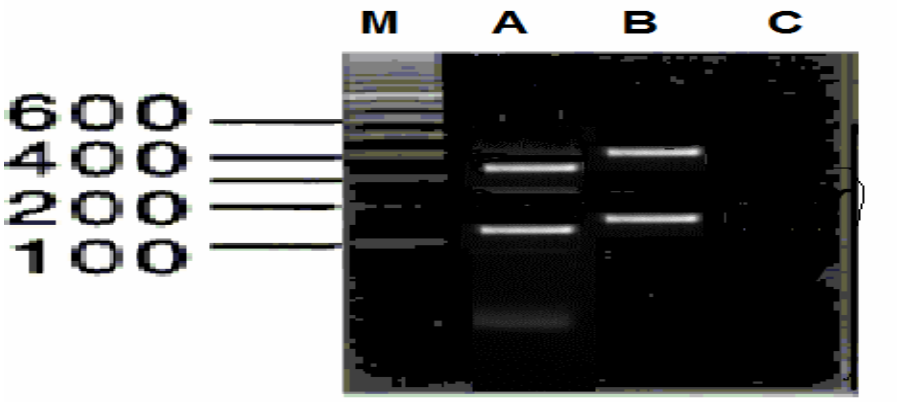

Figure (3) Positve PCR-RFLP for points mutations M=Marker A: point mutations A2143G B: point mutations A2142G

The isolated *H.pylori* in the present study showed marked resistance for clarithromycin (46.2%) and amoxacillin (20.8%) with lower rates of resistance to tetracycline (15.4%) and metronidazole (12.8%).

However The rate of primary resistance reported in a study in Tunisia in 2010 for clarithromycin and metronidazole in Tunisia were respectively 15.4% and 51.3 % (35) high Metronidazole resistance reported in other studies maybe due to its use for gynecological, dental and parasitic related infectious diseases (31), our data suggest lower rate (12.8%) of resistance to metronidazole which favour its use in first line therapy better than amoxacillin. Amoxacillin resistance in the present work was marked (20.8%) can be attributed its uncontrolled use without medical prescription (24). *H. pylori* resistance rates of 97.5%, 72.5%, 66% and 20.5% for amoxicillin have been reported in South Africa, India, Nigeria and Colombia, respectively (31). In general *H. pylori* resistance to tetracycline was detected 11.7% in the world, Tetracycline is extensively used in many countries, but resistance to this antibiotic has not become a great problem yet.

In global studies for *H.pylori* resistance rates from African countries have been reported to be 17.2% for clarithromycin, 26.7% for metronidazole, 11.2% for amoxycillin, 16.2% or tetracycline,(Francesco et al. 2010). Rate of resistance has been reported by other study to be 100% to clarithromycin, ampicillin, and metronidazole in 32 Nigerian patients.

This enormous resistance rates can be attributed to the uncontrolled use of these antibiotics (24). In developed countries, also a high primary and secondary resistance to clarithromycin (49.2 and 70.6%), superior to that seen with metronidazole (32.8 and 41.2%) were reported (25) The great resistance to clarithromycin and amoxicillin in the present study than to other antibiotics can be related to wide use of these antibiotics both in gastrointestinal and respiratory infections. These data should be used to direct the physician to use quadruple therapy with a PPI, bismuth, tetracyclines and metronidazole as an alternative to triple therapy as first-line regimen as the first line regemein is used for population with resistance rates below 15-20%, for clarithromycin and amoxicillin.

Nevertheless, the value of this therapy has been debated due to the increase failure rates. Many factors have been implicated as causes of treatment failure such as ineffective penetration of antibiotics into the gastric mucosa, antibiotic inactivation by the low stomach pH, a lack of patient compliance, and the emergence of acquired resistance to antibiotics by *H. pylori* (26, 27). Therefore, it is recommended to study *H;pylori* resistance pattern in Egypt before considering the first line antibiotics therapy.

The distinguished finding in the present study is the significant increase in presence of mutations in isolates from adults compared to that from children (P=0.006). Generally speaking resistance rates for clarithromycin reflects the annual consumption of macrolides agents among population. It seems that new macrolides description is more common among adults patients than children resulting in more frequent mutations among *H-pylori* strains isolated from adults. This finding is different than that reported by previous study 28).

The most common type of mutation was for A2143G (53.4%) followed by A2142G (35.7%). The mechanism of clarithromycin resistance in *H. pylori* is mainly associated with the following point mutations in 23S rRNA gene: 1) A2143G occurring in 69.8% of strains; 2) A2142G occurring in 11.7% of isolates as previousely mentioned (14; 29). Only 3 isolates could not be identified to have any of the two types of the studied. It can be suggested that this resistance may be associated with less frequent point mutions or even that in these strains other mechanisms which are not related to the 23S rRNA gene sequence, such as the presence of an efflux pump, may play a role in resistance to clarithromycin, as has been described by other authors (30).

The present study concludes from this study that clarithromycin resistance among *H.pylori* isolates is a common finding associated with specific point mutations of A2143G followed by A2142G. Resistance also to other antibiotics amoxicilline, tetracyline are common. Extended studies should be performed on large isolates from Egypt to elucidate the efficacy of first line triple therapyand establish local antibiotic policy for management of H. pylori infection.

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How to cite this article:

Maysaa El Sayed Zaki, Doaa M. Sherif, Mahmoud Abdelwahab Ali, Ahmed Shehta, Ahmed Megahed, Mona Abdel Latif Alsayed, Tarek Barakat and Sheren M Elzeny. 2016. Molecular Study of Clarithromycin Resistant *Helicobacter pylori* strains from Egyptian Centre. *Int.J.Curr.Microbiol.App.Sci.* 5(1): 165-173.
doi: http://dx.doi.org/10.20546/ijcmas.2016.501.014