Fluoroquinolone-Resistant *Helicobacter pylori* strains Isolated from One Egyptian University Hospital: Molecular Aspects

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Received 11 January 2016
Accepted 27 March 2016

Introduction

Helicobacter pylori (H. pylori) are fastidious Gram negative rods responsible for a wide variety of gastrointestinal disorders ranging from simple dyspepsia up to gastric adenocarcinoma [1-5]. Treatment of gastrointestinal disorders caused by *H. pylori* is a combination of antibiotics usually amoxicillin and metronidazole or clarithromycin besides an acid suppressor.

The combination of two antibiotics is used to increase the eradication rates of *H. pylori* and decrease the development of antibiotic resistance [6,7]. The resistance pattern of organisms parallels the patterns of antibiotics consumption in the community. So, the prediction of antibiotics resistance rate among different geographical localities is the main goal of microbiological laboratories to eradicate resistant *H. pylori* strains [8]. There is increasing rates of resistance of *H. pylori* toward clarithromycin reported in various studies [9,10]. This leads to introduction of fluoroquinolones as an alternative substitute to antibiotics used in treatment of *H. pylori* [6,7]. In several studies, the use of fluoroquinolones has provided a successful alternative therapy in persistent *H. pylori* infections [11,12]. The mode of action of fluoroquinolones as bactericidal antibiotic is carried out by the inactivation of deoxyribonucleic acid (DNA) gyrase and topoisomerase enzymes that control

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ABSTRACT

Objective: The aim of the present study was to determine in vitro susceptibility of isolated *Helicobacter pylori* strains to fluoroquinolones by disc diffusion method and by genotypic method using polymerase chain reaction and sequencing for determination of gyrA gene mutations at positions 87 and 91 of the *H. pylori*.

Methods: The study included eighty two adult patients complaining of upper gastrointestinal disorders. Biopsy samples were obtained from patients with endoscope and subjected to microbiological culture for *H. pylori*. Antibiotics susceptibility was determined for the isolates to clarithromycin, amoxicillin, metronidazole, ciprofloxacin and levofloxacin. Strains of *H. pylori* resistant to ciprofloxacin were subjected to study by polymerase chain reaction and sequencing for determination of gyrA gene mutations at positions 87 and 91 of the *H. pylori*.

Results: There were high antibiotic resistance tendencies toward clarithromycin (71%) and amoxicillin (47.8%) among isolated *H. pylori* strains, while resistance patterns were less towards ciprofloxacin (28.9%), levofloxacin (23.2%) and metronidazole (21.7%). GyrA gene mutations were detected in 19 (95%) *H. pylori* isolates resistant to ciprofloxacin with the most common type mutations in codon A at amino acid position Asp-91 in 12 (63.2%) isolates followed by point mutations at amino acid Asn-87 in 7 (36.8%) isolates. Only one resistant *H. pylori* strain toward ciprofloxacin had no detected mutations in gyrA gene.

Conclusions: The present study highlights the high prevalence of *H. pylori* resistant strains to amoxicillin and clarithromycin which thought to be the first line of antibiotics therapy. The study draws attention that fluoroquinolone resistance is less than that for first line therapy which may be a better substitute for first line antibiotics. The mechanism of fluoroquinolone resistance is mainly associated with gyrA gene mutations most commonly in the gyrA protein at position 91.

KEY WORDS: *Helicobacter pylori* Fluoroquinolone *gyrA,* PCR

DNA in cells and interfere with bacterial DNA replication. The structures of these enzymes are formed of two A and two B subunits, encoded by the gyrA and gyrB genes (8). However, even with the use of fluoroquinolone as a new therapy for *H. pylori*, therapeutic failure rates were reported due to the development of bacterial resistance [13-15]. The resistance to fluoroquinolone in *H. pylori* has been attributed to mutations in the quinolone resistance determining regions of the gyrA and the gyrB genes. The most significant mutations conferring quinolone resistance lie at positions 87 and 91 of the *H. pylori* gyrA gene. These findings of *H. pylori* resistance patterns differ according to the geographical region thus making laboratory detection of antibiotics resistance pattern a mandatory tool for *H. pylori* eradication [16]. Laboratory determination of *H. pylori* antimicrobial susceptibility can be performed either by phenotypic or by genotypic methods. Phenotypic methods include disc diffusion method, agar dilution method and E-test [17-20]. The main disadvantage of phenotypic methods is the fact that *H. pylori* are a slow growing organism. On the other hand molecular method is a rapid method. The aim of the present study was to determine in vitro susceptibility of isolated *H. pylori* strains to fluoroquinolones by disc diffusion method and by genotypic method using polymerase chain reaction and sequencing for determination of gyrA gene mutations at positions 87 and 91 of the *H. pylori*.

Materials and Methods

The study included eighty-two adult patients complaining of gastrointestinal symptoms undergoing upper gastrointestinal endoscopes from January 2015 until August 2015. The patients were referred from Gastroenterology Center, Mansoura University, Egypt. The study was approved by the ethical committee of Mansoura Faculty of medicine. Approval consent was obtained from each participating subject. Biopsies were obtained from the greater curvature of stomach about 2 cm from pylorus, and transported to the laboratory on phosphate buffered saline in sterile container. In the lab each biopsy was cultured after homogenization over Columbia blood agar supplied with 5% sheep blood (Oxoid Columbia agar base) and the antibiotic supplements. Plates were incubated at 37ºC up to 2-3 days in a microaerophilic atmosphere by use of gas packs (Campy Pak; Becton Dickinson) in an anaerobic jar. Identification of *H. pylori* was made by Gram staining of the colonies and testing for the presence of urease, oxidase and catalase [21].

Antibiotics susceptibility of *H. pylori*

Antibiotics susceptibility of isolated *H. pylori* strains was performed using the disc diffusion method for amoxicillin, levofloxacin, clarithromycin, metronidazole and ciprofloxacin with preparing inoculums density equivalent of 3.0 McFarland units over Muller-Hinton agar under microaerophilic condition for 72 hours. Pure colonies of *H. pylori* were kept in Schaedler broth at -70°C for further molecular studies for detection of gyrA gene mutations for resistance to fluoroquinolones.

PCR for gyrA gene

Extraction of DNA

Broth culture of *H. pylori* found resistant to ciprofloxacin was taken for extraction of DNA. Extraction was done by the use of QIAamp DNA Mini Kit (Qiagen, USA) according to the manufacturer's instructions.

Amplification of the extracted DNA

Amplification of the eluted DNA was performed in a 50 ul reaction volume for the detected gene. Gyr APF (5′-AGCTTATTCCATGAGCGTGA-3′) and gyr APR (5′-TCAGGCCCTTTGACAAATTC-3′) were designed to amplify a 582 bp amplicon. The PCR mix consisted of 25 µl of master mix containing HotStar Taq DNA Polymerase, PCR buffer (with 3 mM MgCl2), and 400 µM each dNTP, 10 µl of the eluted DNA, 1 µl of each primer (50 pM) of the gene to be detected and 13 µl distilled water. Amplification of DNA was performed in a thermal cycler (thermo PxE 0.21, England) and included initial denaturation for 5 min, followed by 35 cycles of 94℃ for 30 s, 53℃ for 30 s and 72℃ for 30 s, with a final extension step of 72℃ for 10 min. The reaction products were visualized by running 5 µl of the reaction mixture on 1.5% agarose gel [8].

Sequencing

Amplified PCR products were purified by the use of QIA quick PCR purification kit (Qiagen). Later on the purified PCR extracts were subjected to sequence study of gyrA gene with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems,

Germany) using the PCR primers as sequencing primers and the obtained results were analyzed with sequencing analysis software (Genecodes, USA).

Statistical analysis

Data entry and analysis were performed using SPSS version 16. The data were expressed as mean ± SD and percentages.

Results

The study included 82 adult patients complaining of upper gastrointestinal disorders underwent gastroduodenal endoscope examinations. They were 42 (51.2%) male and 40 (48.8%) female with mean age 43±8.5 years. Previous antibiotics therapy for *H. pylori* was reported from 25 (30.5%) patients. Positive culture for *H. pylori* was detected in 69 patients (84.1%), table 1. There were high antibiotic resistance tendencies towards clarithromycin (71%) and amoxicillin (47.8%) among isolated *H. pylori* strains, while resistance patterns were less towards ciprofloxacin (28.9%), levofloxacin (23.2%) and metronidazole (21.7%), table 2. GyrA gene mutations were detected in 19 (95%) *H. pylori* isolates resistant to ciprofloxacin with the most common type of mutations in codon A at amino acid position Asp-91 in 12 (63.2%) resistant isolates followed by point mutations at amino acid Asn- 87 in 7 (36.8%) resistant isolates. Only one resistant *H. pylori* strain toward ciprofloxacin had no detected mutations in gyrA gene (table 3).

Discussion

H. pylori is a common pathogen causing upper gastrointestinal tract disorders especially in less developed countries due to low socioeconomic standards and bad sanitary conditions. With the increasing frequency of resistance among *H. pylori* strains, there is rising concern about the potential decline in the eradication rate of this infection and therefore an urgent need to introduce other treatment options. In the present study culture of *H. pylori* from symptomatic adults yielded high positive rate (84.1%).

Table 1. Demographic data and positive findings of the studied cases.

Descriptive data of the studied cases	
Sex	
Male No. (%)	42 (51.2%)
Female No. (%)	40 (48.8%)
Age (mean±SD) years	43±8.5
Previous therapy No. (%)	25 (30.5%)
Positive culture for *H. pylori* No. (%)	69 (84.1%)

Table 2. Antibiotics resistance pattern among *H. pylori* isolates (n=69).

Antibiotics	**N0. (%)**
Clarithromycin	49 (71%)
Amoxicillin	33 (47.8%)
Metronidazole	15 (21.7%)
Ciprofloxacin	20 (28.9%)
Levofloxacin	16 (23.2%)

Table 3. Frequency of GyrA gene mutations in resistant *H. pylori* isolates to ciprofloxacin (n=20).

GyrA mutations	**N0. (%)**
Mutations	19 (95%)
Point mutations Asp-91	12 (63.2%)
Point mutations Asn-87	7 (36.8%)
No mutations	1 (5.0%)
Total	20 (100%)

These results are in agreement with previous results that stated that infections rates for adults by *H. pylori* ranges from 47.2 up to 90% [5-7,22]. Endoscopy is required for diagnosis of gastrointestinal symptoms especially in patients above the age of 45 years with new upper gastrointestinal complaints [23,24]. It is also a valuable tool to obtain biopsy for microbiological culture and accurate determination of antibiotics susceptibility of *H. pylori*. There have been many reports about therapeutic failure of *H. pylori* due to development of antibiotics resistance. The results from different geographical regions indicate that the effectiveness of therapeutic regimen differs according to antibiotic resistant rates for *H. pylori* which depends upon antibiotics consumption policy [25].

In the present study there were high antibiotic resistance tendencies toward clarithromycin (71%) and amoxicillin (47.8%) among isolated *H. pylori* strains, while resistance patterns were less towards ciprofloxacin (28.9%), levofloxacin (23.2%) and metronidazole (21.7%). Fathi et al., (2013) [26] reported high resistance rates among *H. pylori* isolated from one Egyptian center that constituted 87.5% for amoxicillin, 100% for each of clarithromycin and metronidazole and 12.5% for ciprofloxacin. High rates of resistance towards amoxicillin of isolated *H. pylori* were reported by previous studies that ranged from 19.4% up to 100% (27-30). Also, high rates of *H. pylori* resistance to clarithromycin that ranged from 32% to 45.2% were reported by previous studies [31,32].

Though much lower rates of resistance of *H. pylori* to clarithromycin were reported in other studies that ranged from 14.3% to 17.9% [33,34]. The high rate of clarithromycin and amoxicillin resistance detected in this study is likely to be attributed to an overuse of these antibiotics for the treatment of upper respiratory tract infections and gastrointestinal tract disorders.

In general, the first line therapy for *H. pylori* includes acid suppressors with the use of antibiotics namely clarithromycin and amoxicillin [34]. Metronidazole can be used instead of amoxicillin in certain conditions like in patients with a penicillin allergy due to the increase failure rates of this therapy [35,36]. The use of quinolone or rifabutin is preferred especially after antimicrobial susceptibility testing and in geographical regions where high rates of resistance is anticipative [35,37,38].

The common mutations responsible for quinolone resistance are located at positions 87 and 91 of the *H. pylori* gyrA gene, which encodes the A subunit of the DNA gyrase enzyme [39,40].

Resistance to fluoroquinolones is generally very low (<10%) worldwide. In our study we found a low resistance rate for ciprofloxacin (28.9%) and levofloxacin (23.2%). Lower rates of resistance to quinolones were reported by previous study in Egypt and represented 12.5% by using the same susceptibility testing as in the current study (Fathi et al., 2013) and by other authors [34,41]. The rate reported in this study is lower than that detected by other studies that was high up to 34.5% [31,32].

Our results draw attention towards better treatment of *H. pylori* to guard against chronicity and developments of its complications. It appears that we have to change the therapeutic policies for *H. pylori* by the use of ciprofloxacin and metronidazole. If the culture and antimicrobial susceptibility is available, it will be an appropriate choice to wait for the antimicrobial results.

To study the mechanism of fluoroquinolone resistance in isolated *H. pylori* strains we performed PCR and subsequent sequence study of gyrA region claimed to be responsible for resistance. The mechanism of fluoroquinolone resistance in *H. pylori* has been found to be associated with mutations in the quinolone resistance-determining regions (QRDRs) of the gyrA gene.

Our findings had shown that fluoroquinolone resistance in *H. pylori* is due mainly (95%) to mutations in the QRDR of gyrA with the most common type mutations in codon A at amino acid position 91 was found in 12 (63.2%) resistant isolates followed by point mutations at amino acid 87 in 7 (36.8%) resistant isolates. Only one resistant *H. pylori* strain toward ciprofloxacin had no detected mutations in gyrA gene. It has been reported that the prevalence of mutations rates vary from one country to another [42]. In one study in Hong Kong the most frequent mutation site in the gyrA protein was position 91 [43]. We could not find any studies about the frequency of gyrA mutations in Egypt.

The present study highlights the high prevalence of *H. pylori* resistant strains to amoxicillin and clarithromycin which are thought to be the first line of antibiotics therapy. The study draws attention that fluoroquinolone resistance is less than that for first line therapy which may be a better substitute for first line antibiotics. The mechanism of fluoroquinolone resistance is mainly associated with gyrA mutations most commonly point in the gyrA protein at position 91.

Conflict of Interest

We declare that we have no conflict of interest.

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