The Egyptian Journal of Medical Human Genetics xxx (2018) xxx–xxx

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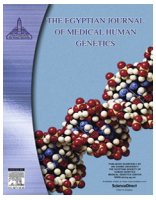
Contents lists available at ScienceDirect

The Egyptian Journal of Medical Human Genetics

journal homepage: www.sciencedirect.com



Original article

Detection of antimicrobial resistance genes of *Helicobacter pylori* strains to clarithromycin, metronidazole, amoxicillin and tetracycline among Egyptian patients

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ARTICLE INFO

Article history:
Received 10 December 2017
Accepted 14 January 2018
Available online xxxx

Keywords:
H. pylori
Antimicrobial resistance
Gene mutations
Rdx gene
Clarithromycin resistance
Tetracycline resistance

ABSTRACT

Background: Antibiotic resistance of *Helicobacter pylori* (*H. pylori*) treatment is on the rise, and is affecting the efficacy of current used therapeutic regimens.
Aim: We aimed to enhance the understanding of antimicrobial resistance rates of *H. pylori* strains recovered from patients at Theodor Bilharz Research Institute Hospital in Egypt, as a mandatory step before starting treatment.
Subjects and methods: Mutant genes conferring metronidazole, amoxicillin, clarithromycin, and tetracycline resistance were detected in 60 *H. pylori* strains recovered from patients who underwent upper endoscopic examination. Patients were considered to be infected with *H. pylori* when rapid urease test and detection of *16S rRNA* in gastric biopsies recorded positive. Molecular detection of resistant genes to metronidazole (*rdx* gene) and amoxicillin (*pbp1A* gene) was carried out by conventional PCR followed by sequencing of PCR products. While detection of *23S rRNA* gene conferring clarithromycin resistance and detection of *16S rRNA* mutation gene conferring tetracycline resistance were carried out by real-time PCR.
Results: H. pylori resistance rates to metronidazole, and amoxicillin were 25% and 18.3% respectively. While for clarithromycin and tetracycline, point mutations in *23S rRNA* types *A2142G* and *A2143G* and in *16S rRNA* of *H. pylori* were assessed by real time PCR assay respectively. Resistance mutant genes were found to be 6.7% of clarithromycin and 1.7% of tetracycline. Combined resistance rates to metronidazole and amoxicillin was (11.6%) followed by metronidazole and clarithromycin (5%), while patterns of clarithromycin and amoxicillin (1.6%), metronidazole, clarithromycin and amoxicillin (1.6%) were revealed.
Conclusion: Data concerning antimicrobial resistance genes play an important role in empiric treatment of *H. pylori* infection. According to our results, *H. pylori* resistance to metronidazole and amoxicillin was relatively high. Clarithromycin is still a good option for first line anti-*H. pylori* treatment. Combined resistant strains are emerging and may have an effect on the combination therapy.

1. Introduction

Eradication of *H. pylori* does not only heal gastritis of peptic ulcer disease, but may prevent the spread of infection and *H. pylori* recurrence. Also, it may reduce the risk of development of gastric cancer, thus reducing further costs required for the treatment of subsequent *H. pylori*-associated diseases [1]. The treatment of *H. pylori* infection employs a triple drug regimen using one of the following antibiotics (amoxicillin, tetracycline or clarithromycin)

Abbreviations: H.pylori, *Helicobactor pylori*; PPI, Proton pump inhibitor; TBRI, Theodor Bilharz Research Institute; DNA, Deoxy-ribonucleic acid; PCR, Polymerase chain reaction; *16S rRNA*, 16 S ribosomal ribonucleic acid; dNTPs, Deoxynucleotide Triphosphates; SPSS, Statistical Package for Social Sciences; Bp, Base pair; Lab, Laboratory; Ul, Micro-litre; min, Minute; UV light, Ultraviolet light; Pmol, Picomole; Tm, Temperature.

Peer review under responsibility of Ain Shams University.

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https://doi.org/10.1016/j.ejmhg.2018.01.004
1110-8630/

along with metronidazole and a proton pump inhibitor (PPI) or bismuth salt, and combined PPI and bismuth salt with quadruple regimen when triple therapy regimens have failed. In Egypt, the standard therapy combines PPI and metronidazole, and one antimicrobial drug, chosen from among clarithromycin and amoxicillin [2–4].

Antibiotic resistance is a key factor in the failure of eradication therapy and recurrence of *H. pylori* infection [5]. Worldwide *H. pylori* antibiotic resistance towards different antibiotics is increasing and it is the main factor affecting efficacy of current therapeutic regimens. Prevalence of bacterial resistance varies in different geographic areas, and it has been correlated with the widespread use of certain antibiotics in the general population (i.e. clarithromycin for respiratory infections, metronidazole for parasite diseases and dental infections, tetracycline for respiratory and bowel diseases, amoxicillin for streptococcal pharyngitis, urinary tract infections [6]. In countries where the use of clarithromycin is rare, the resistance rate is low, while in countries where its use is wide, the resistance rate reaches 10–15%. Similarly, metronidazole-resistance rate is much higher in developing countries (50–80%) [7]. Amoxicillin is suggested for anti-*H. pylori* triple therapy in regions where metronidazole resistance is high. Universal resistance to amoxicillin is uncommon; it was detected in 14.67% [8].Tetracycline resistance has not become a great problem yet [2].

The aim of this study was to enhance the understanding of antimicrobial resistance rates of *H. pylori* strains recovered from patients at Theodor Bilharz Research Institute TBRI Hospital in Egypt, as a mandatory step before starting treatment.

2. Subjects and methods

2.1. Patients and clinical specimens

From December 2015 to January 2017, 60 *H. pylori* infected patients who underwent upper endoscopy for various dyspeptic symptoms at Endoscopy Unit, TBRI Hospital were enrolled in this study. The sample size was calculated by Epi Info program (version 6.0) at 95%Confidence Limit, Power of the Test is 80% and Alpha Setat 0.05 (Type Error). None of the patients had received non-steroidal anti-inflammatory drugs, as well as antibiotics, H_2 receptors antagonists or proton pump inhibitors in the past four weeks prior to the study. During upper gastrointestinal endoscopy, four antral biopsies were obtained from each patient. One biopsy was tested for rapid urease test, that was performed using rapid urease liquid test kit (Bussero, Milan, Italy) and the other three gastric biopsies were stored in sterile physiological saline and kept at −70 °C until processed. DNA extraction used directly for detection of *16S rRNA*, *rdxA, Pbp1*, *23S rRNA* and *16S rRNA* mutation genes using PCR assays. A patient was considered to be infected with *H. pylori* when he had positive rapid urease test and confirmed by detection of *16S rRNA* in gastric biopsy specimens. This work was supported by Theodor Bilharz Research Institute (TBRI) as a part of an ongoing internal project No. 93 T. The protocol was approved by TBRI institutional review board (FWA00010609) and all patients provided a written informed consent. The work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for Experiments in Humans.

2.2. DNA extraction from gastric biopsy specimens and PCR assays

Genomic DNA was extracted from gastric biopsy specimens using QIAamp tissue kit (QIAamp DNA Mini Kit from QIAGEN, USA), following manufacturer guidelines.

2.2.1. Detection of H. Pylori 16S rRNA gene

Conventional PCR assay was performed in a volume of 50ul with approximately 5 μg of extracted DNA, 200 μM (each) dNTPs, 25 pmol for each primer, 1.5 μM Magnesium Chloride and 1unit (U) of *Taq* polymerase (Gotaq Flexi DNA, M8305, Promega, Inc., USA) in PCR buffer using primers according to[9]. The sequences of forward (F) and reverse (R) primers are: F 5′-CTG GAG AGA CTA AGC CCT CC-3′ and R 5′-ATT ACT GAC GCT GAT TGT GC-3′.

An initial denaturation at 95 °C for 5 min followed by 35cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min and 72 °C for 1 min. This was followed by a final extension at 72 °C for 5 min. The size of the expected amplicon was 110 bp.

2.2.2. Detection of rdxA gene conferring metronidazole resistance

Conventional polymerase chain reaction (PCR) assay was performed in a volume of 50ul with approximately 2.5 μg of the extracted DNA, 200 μM (each) dNTPs, 2.5 μM Magnesium Chloride and 0.25 U of *Taq* polymerase (Promega®, USA) in PCR buffer using 25 pmol primers. Primer sequences according to (Debets-Ossenkopp et al. [10] are: F: 5′-AAT TTG AGC ATG GGG CAG A-3′ and R: 5′-GAA ACG CTT GAA AAC ACC CCT-3′.

Amplification was carried using; denaturing temperature at 95 °C for 5 min, followed by 40 cycles denaturing temperature at 94 °C for 30 s, an annealing temperature at 55 °C for 30 s and an elongation temperature at 72 °C for 1 min. This was followed by a final extension of 72 °C for 10 min. The size of expected amplicon was 581 bp.

2.2.3. Detection of Pbp1A gene conferring amoxicillin resistance

Conventional PCR assay was performed in a volume 50ul, with approximately 1 μg of extracted DNA, 200 μM (each) dNTPs, (Table: 3), 0.5u of *Pfu* DNA polymerase (Thermo®, USA) in PCR 5 μl *Pfu* buffer with Magnesium Sulphate and 25 pmol primer [12]. The sequences of the F and R primers are: F 5-GCG ACA ATA AGA GTG GCA-3′ and R 5′-TGC GAA CAC CCT TTT AAA T-3′.

The following cycling parameters were used; denaturing temperature of 95 °C for 3 min, followed by 35 cycles of denaturing temperature of 95 °C for 1 min, an annealing temperature of 54 °C for 1 min and an elongation temperature of 72 °C for 5 min and final extension at72°C for 10 min. The size of expected amplicon was 2300 bp.

Each PCR product was separated on 2% agarose gel with ethidium bromide, and 100 bp ladder used as DNA molecular weight standard. In each PCR assay, a negative control (lacking DNA) was included. PCR products were analyzed under UV light [11].

2.2.4. Sequencing of DNA fragments of rdxA and Pbp1A genes

Genomic DNA fragments were purified from agarose gel using purification kit (Thermo Scientific GeneJET Gel Extraction Kit, USA) according to the manufacturer's instructions. DNA sequencing was made by Sanger sequencing method following the manufacturer's instructions. Based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication [12].

To compare between wild and mutant genes, alignment was made online at site http://www.ebi.ac.uk/Tools/msa/clustalw2/.

2.2.5. Detection of 23S rRNA gene conferring clarithromycin resistance

The real-time (TaqMan, USA) PCR assay was performed in a volume of 25ul, with approximately 10 ng of purified DNA, 12.5 μl universal PCR master mix (ABI, USA), 36 ul of each primer, 8 μM of FAM- and VIC-labeled TaqMan MGB probes [13].

Primers and probes sequences according to Lins et al. [14] are:
F: 5′ TCA GTG AAA TTG TAG TGG AGG TGA AAA-3′
R: 5′ CAG TGC TAA GTT GTA GTA AAG GTC CA-3′
VIC