RESEARCH ARTICLE

Real-Time PCR Detection of 16S rRNA Novel Mutations Associated with *Helicobacter pylori* Tetracycline Resistance in Iran

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Abstract

Background: Tetracycline is an antibiotic widely used for the treatment of *Helicobacter pylori* infection, but its effectiveness is decreasing due to increasing bacterial resistance. The aim of this study was to investigate the occurrence of 16S rRNA mutations associated with resistance or reduced susceptibility to tetracycline of*Helicobacter pylori* by real-time PCR (RT-PCR) assays from culture. Materials and Methods: Tetracycline susceptibility and minimal inhibition concentration (MIC) was determined by the Epsilometer test (Etest) method. A LightCycler assay developed to detect these mutations was applied to DNA extracted from culture. The 16S rRNA of these isolates was sequenced and resistance-associated mutations were identified. From 104 isolates of *H. pylori* examined, 11 showed resistance to tetracycline. Results: LightCycler assay was applied to DNA extracted from 11 tetracycline-susceptible and 11 tetracycline resistance *H. pylori* isolates. In our study the sequencing of the *H. pylori* wild types in 16 s rRNA gene were AGA 926-928 with MIC (0.016 to 0.5μg/ml), while the sequencing and MIC for resistant were GGA and AGC, (0.75 to 1.5μg/ml), respectively. Also we found a novel mutation in 2 strains with 84°C as their melting temperatures and exhibition of an A939C mutation. Conclusions: We conclude that real-time PCR is an excellent method for determination of *H. pylori* tetracycline resistance related mutations that could be used directly on biopsy specimens.

Keywords: Antimicrobial resistance mechanisms - light cycler - DNA polymorphisms

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Introduction

Helicobacter pylori infection is chronic situation that increases the risk of development of peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma (MALT), and gastric cancer. In many developing countries, such as Iran, *H. pylori* infect approximately 80 % of the population. Management of *H. pylori* infections is difficult, and relapse frequently occurs after successful eradication of the organism (Malfertheiner et al., 2007, MalekiMSc and SiavoshiPhD, 2013). Increasing rates of resistance to the antibiotic such as metronidazole and clarithromycin are result in therapy failures. Therefore, alternative treatments that include tetracyclines are recommended (Milani et al., 2012; Mansour-Ghanaei et al., 2013; Wang et al., 2014).

Tetracyclines are bacteriostatic drugs which affecting the 30S subunit of the ribosome and block the binding of aminoacyl-tRNA, resulting in impaired protein biosynthesis (Gerrits et al., 2002). The tetracycline resistance to *H. pylori* is reported to be caused by mutations in the 16S rRNA. Although in worldwide tetracycline resistance remains relatively uncommon. However, in regions where tetracycline is more widely used in *H. pylori* therapy, evidence has emerged of increasing resistance. For example, reported resistance levels were 2.7% in America, 2.1% in Europe, 2.4% in Asia, 43.9% in Africa (De Francesco et al., 2010) and 9% in Iran (Talebi Bezmin Abadi et al., 2010). The susceptibility of *H. pylori* to tetracycline is routinely examined by agar diffusion (Etest) or agar dilution tests, which are accepted to be the "gold standards" (Heep et al., 2000). These methods are slow and time-consuming, and occasionally fail due to a lack of growth of *H. pylori* or due to overgrowth with other bacteria. It has already been shown that real-time PCR assays are excellent methods for prediction of resistance to tetracycline in *H. pylori* (Glocker et al., 2005a). In this study, we report the occurrence of 16S rRNA mutations associated with resistance and reduced susceptibility to tetracycline in *H. pylori* isolates from Iran and describe a real-time PCR assay for the rapid detection of resistance.

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Materials and Methods

Bacterial strain and determination of susceptibility to tetracycline:

A total of 104 *H. pylori* isolates were obtained from gastric biopsies of patients with gastritis, peptic ulcer disease (PUD) and astroesophageal reflux diseases (GERD) undergoing endoscopy. This study was approved by the ethical committee of regional Medical Research of Tabriz University of Medical Sciences and all patients provided written informed consent for this research.

Briefly all gastric biopsies were cultured in Burcella agar (Pronadisa, Spain) and incubated under microaerophilic conditions at 37°C for 5 to 7 days. Organisms were identified as *H. pylori* on the basis of colony morphology, Gram staining, and positive oxidase, catalase, and rapid urease tests (McNulty et al., 2002). The disk diffusion and Etest methods (Liofilchem, Italy) were used to determine the susceptibility and MIC of tetracycline. Strains were classified as resistant when the MIC was ≥1μg/ml and as sensitive when the MIC was <1μg/ml (Abrams et al., 2000).

DNA extraction and 16S rRNA gene amplification:

Genomic DNA from the 11 isolates with reduced susceptibility or resistance to tetracycline (MIC≥0.5μg/ml) and the 11 tetracycline-susceptible (MIC<0.5μg/ml) *H. pylori* isolates were extracted using a QIAmp DNA mini kit (QIAGEN). As PCR product, 120 bp fragment of *H. pylori* 16S rRNA genes was amplified and sequenced using the primers 16S Fw (5'-ATAGACGGGGACCCGCACAAG-3') and Rv (5'-TGGCAAGCCAGACACTCCA-3') as published literature (Glocker et al., 2005a).

DNA Sequencing:

The 16S rRNA sequences of 22 isolates (including resistance, insusceptible and susceptible) were determined. *H. pylori* 16S rRNA gene was amplified and sequenced using primers described above then DNA sequencing was done using the CEQ dye terminator cycle sequencing quick start kit run on a Beckman (Beckman Coulter, USA).

Detection of 16S rRNA gene mutations of H. pylori by real-time-PCR:

Real-time PCR was performed on bacterial DNA extracted from isolates. The method included the amplification of the 16S rRNA gene fragment of *H. pylori* using the primers mentioned above and the simultaneous detection of the PCR-product by hybridization probes. For the detection of the 16S rRNA gene mutations, two hybridization probes, an anchor-probe 16S-Anc (5'-TCT AGC GGA TTC TCT CAA TGT CAA GCC TAG-3'; 3' labeled with fluorescein) covers nucleotides 975 to 946 and a mutation-probe 16S-AGA-Sensor (5'-AAG GTT CTT CGT GTA TCT TCG-3'; 5' labeled with Light Cycler Red640 and 3' phosphorylated) covers nucleotides 943 to 923 were used. Also additional probe, 16S-TTC-Sensor (5'-AAG GTT CTT CGT GTA GAA TCG-3'; 5' labeled with Light Cycler Red705 and 3' phosphorylated) that matched the sequence of the TTC mutant.

Triplicate real-time PCRs were performed in 20μL-volumes in microtube using a LightCycler. Twenty microliters of PCR mixture contained 10μL of SYBR-Green Master Mix (Qiagen), 0.4μL of each of the primers Hp16S-F1 and Hp16S-R1 (25μM each), 2μL of the anchorprobe and 2μL of the mutation-probe (2μM each), 3.2μL of H2O and 2μL of template DNA. Cycling conditions consisted of an initial denaturation step of 95°C for 15 min, followed by 32 cycles with denaturation at 95°C for 20 sec, annealing at 54°C for 20 sec and an extension step at 72°C for 20 sec. Melting curves were plotted automatically and analyzed with the LightCycler-software.

Results

A total of 104 *H. pylori* isolates were screened and 8 TetR isolates were obtained with MICs of 1-1.5μg/ml. According to previous study, *H. pylori* isolates were classified as resistant to tetracycline when the MIC was ≥1μg/ml (Glocker et al., 2005b). Therefore all isolates were classified as susceptible, insusceptible or resistance. Our study revealed 11 isolates (10.6%) resistance to Tetracycline using the disk diffusion method (with 30 ≥ mm inhibition zone), while 9 isolates (8.7%) were resistant with MICs ranging from 1-1.5μg/ml according to the E-test method. Out of eleven isolates, three strains showed MIC=1.5μg/ml, five strains had MIC=1μg/ml and three isolates with MIC= 0.75μg/ml. In this study the LightCycler assay was applied to DNA extracted from 11 tetracycline-susceptible, 9 resistance and 2insusceptible *H. pylori* isolates.

In our study the sequencing of the *H. pylori* wild types in 16s rRNA gene were AGA 926-928 with MIC (0.016 to 0.5μg/ml), while the sequencing and MIC for other isolates foresaid were as followed (Table 1).

As shown in table 1, isolates carrying mutation in 16s rRNA gene showed different Tm (melting temperature) based on types and regions of mutation.

Screening for mutations associated with tetracycline resistance:

Upon analysis of the melting curves of all resistance and insusceptible tetracycline isolates, two melting temperatures corresponding to two different genotypes were found: 5 strains showed melting temperatures of 83.9-84°C and exhibited an A926G mutation. For two isolates we found a melting temperature of 87.35°C and exhibited an A928C mutation while for two isolates like all wild type (AGA) sequences we found a melting

Table 1. 16S rRNA wild Type and Mutants, MICs of Tetracycline and Melting Temperatures of the 16S rRNA Gene Hybridization Probes

H. pylori isolates	Sequencing (926-928)	Sequencing (939)	Tm (°C) (μg/ml)	MIC range
Susceptible (11 isolates)	AGA	-	88.75	0.016 - 0.5
Resistance (5 isolates)	GGA	-	84	1 - 1.5
Resistance (2 isolates)	AGC	-	87.35	1.5
Insusceptible (2 isolates)	AGA	-	88.75	0.75 - 1
Resistance (2 isolates)	-	GAC	84	1

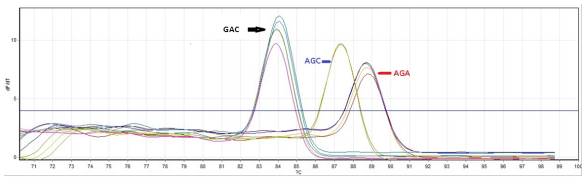

Figure 1. Melting Curve Analysis for Mutations Related with Tetracycline Resistance in 16s rRNA Gene. AGA: wild type, AGC: mutated (A928C), GAC: novel mutation (A939C)

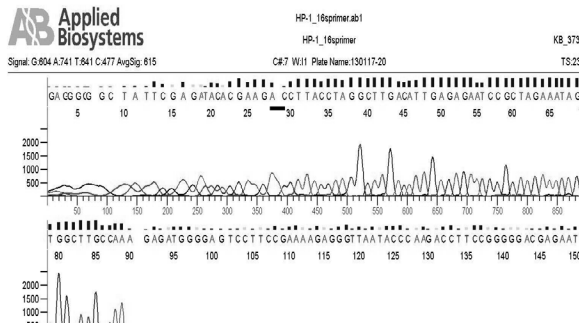

Figure 2. DNA sequencing of the 16s rRNA in three strains with novel mutation for the A939C mutation. This is forward sequence and the position of mutation point indicated with underlying

temperature of 88.75°C as confirmed by subsequent sequencing analysis. Surprisingly in this research we found a novel mutation in 2 strains with 84°C as their melting temperatures and exhibition an A939C mutation (Figure 1 and 2) which MIC with ranging from 0.5-1μg/ml.

Discussion

Due to the emergence multidrug resistance of *H. pylori* to common antimicrobial agents in Iran (Milani et al., 2012) and other Asian countries (De Francesco et al., 2010), alternative treatment include tetracycline might be used more often. Although Tet resistance is rare in *H. pylori* than in other bacteria, there are many differences in geographic areas. For example, in Western countries, Tet resistance is rare (Dailidiene et al., 2002), but in Japan and Korea, 5-7% resistance has been observed, and in China high levels of resistance (59 %) have been found (Wu et al., 2000; Anoushiravani et al., 2009). Recent studies have been reported successful eradication of *H. pylori* by the use of tetracycline-based treatment (Gisbert and Pajares, 2001; Zullo et al., 2012).

Of course the more frequent use of tetracycline is likely to be associated with increasing Tet resistant *H. pylori* isolates in the future. So, it is very important to evaluate for the potential loss of its efficacy due to resistance to this agent.

According by Gerrits et all. results, resistance to tetracycline antibiotics group has been associated with mutation in 16s rRNA gene (Gerrits et al., 2002). They found that transformation of *H. pylori* strain 26695 (MIC, 0.19μg/ml) with genomic DNA of strain 181 (MIC, 8μg/ml) resulted in Tet resistant colonies.

In this study a LightCycler assay successfully distinguished four distinct 16S rRNA mutations associated with resistance and reduced susceptibility to tetracycline from isolates with the wild-type allele. Our study revealed 7 of the 11 isolates with reduced susceptibility to tetracycline had mutations in the 16S rRNA coding for the helix 31 region of 16S rRNA.

The A926G mutation was found in five isolates with MIC that ranged between 1 to1.5μg/ml. This mutation had previously been reported amongst two El Salvador isolates with MIC of 2μg/ml (agar dilution method) and one Germany isolate with MIC of 1μg/ml (Etest method) (Glocker et al., 2005a) and seven UK isolates with MIC of 0.75 to 4μg/ml (Etest method) (Lawson et al., 2005).

Also in our study the A928C mutation was found in two isolates with an Etest MIC of 1.5μg/ml. This mutation had previously been reported in a Lithuanian isolate with MIC of 2μg/ml (agar dilution method) and one isolate from UK with MIC of 1μg/ml (Etest method) (Lawson et al., 2005).

As mentioned before, in this study we found a new mutation in two strains that had not been previously reported. It seems that the occurrence of this mutation alone does not cause a high-level of resistance to Tet (MIC of these strains were 1μg/ml). Maybe if the two mutations occurred simultaneously in one strain, we saw a high level of resistance to this antibiotic.

According by previous studies triplet mutations were resulted increase MIC and cause decrease melting temperature (Glocker et al., 2005a). Our study revealed that wild-type isolates compared with mutant isolates have a higher Tm. Also the results showed that the location and mutation type has affected on the MIC of tetracycline and Tm. Based on the results of this study, any polymorphic and changes in the probe region, resulting in a significant decrease in Tm, which is possible to discriminate it from the wild-type allele. The aim of additional probe design, was more accurate identification of specific mutation in 16s rRNA.

In this study we also found insusceptible tetracycline isolates without mutations in gene 16 s rRNA that had equal Tm as wild type isolates, therefore this indicates that in our *H. pylori* isolates resistance to tetracycline is probably multi-factorial such as activation of efflux pump and mutations in other coding DNAs related to tetracycline.

This method is able to detect strains that are resistant due to mutations. Nevertheless the possible limitation of this method is low sensitivity in diagnosis mutations related to resistance in populations mixed with wild type isolates. However the 16S rRNA polymorphisms are excellent targets for the real-time PCR detection assays that allow assess susceptibility testing direct from biopsies specimen without culture. This method could be especially useful in the patients who have experienced treatment failure. Because biopsy specimens containing damaged *H. pylori* that might be difficult to culture can be examined quickly and directly.

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