

Tetracycline resistance in Chilean clinical isolates of *Helicobacter pylori*

Héctor Toledo* and Remigio López-Solís

Programa de Biología Celular y Molecular, ICBM, Facultad de Medicina, Universidad de Chile, Avenida Independencia 1027, Casilla 70086, Santiago-7, Chile

*Corresponding author. Tel: +56-2-978-6053; Fax: +56-2-735-5580; E-mail: htoledo@med.uchile.cl

Received 20 July 2009; returned 11 August 2009; revised 23 November 2009; accepted 24 November 2009

Objectives: Since high-level tetracycline resistance in *Helicobacter pylori* has been associated with a AGA926–928→TTC substitution in the 16S rRNA genes *rrnA/B*, the aim of the study was to screen for tetracycline resistance in *H. pylori* clinical isolates obtained from Santiago, Chile by using a recently reported molecular assay.

Methods: A PCR-restriction fragment length polymorphism (PCR-RFLP) assay of the conserved 535 bp region of the *H. pylori* 16S rRNA genes *rrnA/B* (between nucleotides 710 and 1245) using HinfI was followed by DNA sequencing of the same fragment obtained from tetracycline-resistant *H. pylori* clinical isolates.

Results: The PCR-RFLP assay revealed that the tetracycline-resistant *H. pylori* isolates lacked the AGA926–928→TTC substitution. In contrast, DNA sequencing of the 535 bp PCR fragment from 11 tetracycline-resistant *H. pylori* Chilean clinical isolates showed an association of low-level tetracycline resistance with 1 bp (A928C) or 2 bp (AG926–927→GT and/or A926G/A928C) substitutions in both 16S rRNA genes.

Conclusions: The PCR-RFLP (HinfI) assay alone is unreliable for the detection of tetracycline resistance in Chilean clinical isolates of *H. pylori*. To that end, it must be complemented by sequencing of the 535 bp PCR fragment.

Keywords: nucleotide substitutions, PCR-RFLP, 16S rRNA gene

Introduction

Helicobacter pylori is a neutrophilic Gram-negative spiral-shaped bacterium that is able to persistently colonize the human stomach. *H. pylori* infection is chronic in nature, causes a serious transmissible infectious disease that damages gastric structure and function, and is recognized as the causative agent in gastric atrophy, peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma.¹ Gastric cancer is the second leading cause of cancer-related death worldwide. It has been estimated that *H. pylori* infection increases the risk of gastric cancer, and the WHO's International Agency for Research on Cancer (IARC) has classified the bacterium as a 'class I carcinogen'. Successful treatment of *H. pylori* infection not only results in the eradication of the pathogen but it often cures and prevents associated diseases.² Most of the current anti-*H. pylori* therapies are highly effective, but the eradication rate of the microorganism is negatively affected by the increasing incidence of antibiotic resistance.³ Drug resistance represents a major issue in the management of *H. pylori* infection. Since triple and quadruple resistant strains have been nearly exclusively isolated from treated patients, unsuccessful antimicrobial eradication seems to represent a major risk factor for the development of multiple resistance.^{3,4} *In vitro*,

H. pylori is susceptible to a wide range of antibiotics but a combination of these drugs is required for an effective treatment of the infection.⁴ Tetracycline-based combination regimens are often used following the failure of first-line treatments with amoxicillin, clarithromycin and metronidazole.^{5,6}

Tetracycline inhibits bacterial growth by blocking protein synthesis. More specifically, this antibiotic binds to the 30S ribosomal subunit by interacting with the 16S rRNA.^{7,8} Most *H. pylori* isolates are still susceptible to tetracycline (MIC < 1 mg/L).^{3,4} However, the incidence of tetracycline resistance has increased in geographic regions where the antibiotic can be obtained without prescription.⁹ Recent epidemiological evidence from Santiago, Chile has shown that after 60 years of widespread antibiotic consumption tetracycline resistance among clinical isolates of *H. pylori* is almost 30%.¹⁰ Tetracycline resistance in *H. pylori* has been mostly associated with mutations in the 16S rRNA gene.¹¹ In two previously described tetracycline-resistant *H. pylori* isolates, high-level tetracycline resistance was found to be associated with the triple base pair substitution AGA926–928→TTC in the 16S rRNA genes *rrnA/B*.^{11,12} Those studies were based on the identification of a second cleavage site by a PCR-restriction fragment length polymorphism (PCR-RFLP) assay with the restriction enzyme HinfI. Using that assay followed by DNA sequencing, in the present study we

conducted a screen of nucleotide substitutions in the same region of the 16S rRNA gene among 41 Chilean clinical isolates tested for tetracycline susceptibility. Here we report that in Chilean tetracycline-resistant *H. pylori* clinical isolates, low-level tetracycline resistance is due to single or double base pair substitutions at positions between 926 and 928 in the 16S rRNA genes *rnaA/B*.

Materials and methods

Patients

Sixty urease-positive patients with no history of previous triple therapy and subjected to endoscopy at the Gastroenterology Unit-Clinical Hospital of the University of Chile were enrolled in the study between June 2005 and October 2006. A written informed consent, which was approved by the Ethics Committee of the Clinical Hospital of the University of Chile, was obtained from all the patients. Three biopsies, two from the antrum and one from the corpus, were obtained from each patient. One of the antrum specimens was used for the rapid urease test and the other two were used for bacterial culture and antibiotic susceptibility testing. *H. pylori* isolates were recovered from 41 patients in the study population.

Growth conditions of bacterial isolates and antibiotic susceptibility testing

Bacteria were routinely grown on trypticase soy agar plates supplemented with 5% horse serum (HyClone, Logan, UT, USA), culture supplement Vitox (Oxoid, Basingstoke, UK) and antibiotic supplement Dent (Oxoid) for 24 h at 37°C in an atmosphere of 5.5% CO₂ and 85% humidity.¹³ The MIC of tetracycline was determined by an agar antibiotic dilution method (0, 0.125, 0.25, 0.5, 0.75, 1.0, 1.25, 1.50, 1.75, 2.0, 4.0, 8.0 and 16.0 mg/L). Isolates were considered to be resistant when the MIC of tetracycline was ≥ 1 mg/L.^{3,4} Routinely, in all these determinations tetracycline-susceptible *H. pylori* strain 26695 served as a control.

DNA extraction and PCR amplification

DNA extraction from cultured isolates was performed using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). The conserved 535 bp region of the *H. pylori* 16S rRNA gene between nucleotide positions 710 and 1245 (numbering according to the *rnaA* gene of *H. pylori* strain 26695)¹⁴ (<http://cmr.jcvi.org/tirg-scripts/CMR/GenomePage.cgi?database=ghp>) was amplified using forward primer 5'-CTGAC GCTGATTGCGCGAA-3' and reverse primer 5'-TGGCTCCACTTCGCAGTATT-3'. PCR conditions were: one cycle at 98°C for 3 min, followed by 30 cycles at 98°C for 1 min, 54°C for 1 min and 74°C for 1 min, followed by a final elongation step at 74°C for 15 min, using ThermoAce™ DNA Polymerase (Invitrogen, Carlsbad, CA, USA). The procedure was performed in a PTC-100 MJ Research thermal cycler. Amplified fragments were visualized on ethidium bromide-stained 2% agarose electrophoresis gels.

RFLP and sequence analysis

To detect the AGA926-928→TTC substitution, PCR products were digested with HinfI (New England Biolabs, Beverly, MA, USA).¹² The restriction products were analysed by electrophoresis on 2% agarose gels, and visualized by ethidium bromide staining. PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA) and subjected to sequencing reactions using the ABI PRISM Big Dye™ Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied

Biosystems, Foster City, CA, USA) and an ABI-3100 machine (Applied Biosystems). All analyses were performed in duplicate.

Results and discussion

Tetracycline is an antibiotic that is frequently used in several second-line regimens for *H. pylori* infection.^{5,6} Besides its wide availability, another major advantage of tetracycline is that, until recently, resistance of *H. pylori* to this antimicrobial drug was rare. High-level tetracycline resistance in *H. pylori* is a serious concern as it negatively affects the efficacy of tetracycline-containing regimens.^{6,15} Previous reports have shown that in two unrelated high-level tetracycline-resistant *H. pylori* strains, tetracycline resistance is conferred by an identical triple base pair substitution (AGA926-928→TTC) in both copies of the 16S rRNA gene.¹¹ Subsequently, several low-level tetracycline-resistant isolates were described and in some of them resistance was mediated by single or double base pair mutations in exactly the same region.¹⁶ In the present study we found that as many as 11 out of 41 Chilean clinical isolates displayed MIC values of tetracycline in the range of 2 mg/L (low-level tetracycline resistant) while the MIC range for the other 30 isolates was from 0.125 to 0.5 mg/L (tetracycline susceptible). Thus, the observed frequency of low-level tetracycline resistance in our study is much higher than those observed recently in several studies worldwide.^{16,17} Conventional methods to assess antibiotic resistance in *H. pylori* are based on culture in combination with either agar antibiotic dilution or the Etest.^{10,18} Both methods are time consuming and do not always yield results that are uniformly interpreted. Nucleic acid-based methods nowadays represent an advantageous alternative because of reduced time to diagnosis and accurate, reproducible and reliable results. Since tetracycline resistance in *H. pylori* has been largely associated with the occurrence of specific mutations in a small region of the 16S rRNA, molecular methods have been proposed for identifying the resistance phenotype. In a recently reported molecular approach for clinical purposes, a 535 bp fragment, corresponding to positions 710-1245 of both *H. pylori* 16S rRNA genes, was amplified by a single PCR followed by digestion with HinfI.¹² The *H. pylori* 26695 reference strain and tetracycline-susceptible *H. pylori* clinical isolates present a single HinfI (5'-G↓ATTC-3') cleavage site within the conserved 535 bp region, thus generating 254 and 281 bp HinfI digestion products. In contrast, mutant strains carrying the AGA926-928→TTC substitution in both copies of the 16S rRNA genes, which mediates high-level tetracycline resistance, display an additional HinfI restriction site in the conserved 535 bp region, thus generating 40, 214 and 281 bp digestion products after incubation with the endonuclease. With this assay, mutants bearing single or double DNA substitutions at positions 926-928 displayed a single cleavage and all of them were reported to be associated with low-level tetracycline resistance.¹⁹

As expected, in our study the tetracycline-susceptible *H. pylori* isolates and the reference *H. pylori* strain 26695 displayed a single cleavage site in the PCR-derived fragment from 16S rDNA, which results in 281 and 254 bp digestion products after incubation in the presence of the restriction enzyme HinfI. On the other hand, when each of the 11 Chilean low-level tetracycline-resistant isolates was subjected to the assay the

Table 1. 16S rDNA mutations that confer tetracycline resistance among Chilean *H. pylori* isolates

Base pair substitutions	Number of tetracycline-resistant isolates
A928C	1
AG926–927→GT	6
AGA926–928→GGC	4

The conserved 535 bp region of both *H. pylori* 16S rRNA genes (nucleotides 710–1245) from 11 tetracycline-resistant *H. pylori* clinical isolates displaying a tetracycline-susceptible PCR-RFLP/HinfI pattern were sequenced and compared with the equivalent region from 10 random tetracycline-susceptible *H. pylori* clinical isolates and from the tetracycline-susceptible *H. pylori* reference strain 26695.

same digestion profile was obtained, that is to say HinfI did not cut the PCR-derived fragment at positions 926–928 to produce 214 and 40 bp fragments. In order to confirm the absence of a second cleavage site at positions 926–928 among the Chilean tetracycline-resistant isolates we performed DNA sequencing studies. These studies showed that the 535 bp PCR-derived fragment from each of the 11 Chilean low-level tetracycline-resistant isolates displayed single or double base pair substitutions (A928C, AG926–927→GT and A926G/A928C) (Table 1). Some of the point mutations identified in this study (AG926–927→GT and A926G/A928C) were different from those previously reported.¹⁹ The triple substitution (AGA926–928→TTC) did not occur. No substitutions were observed in the 535 bp PCR fragment from any of the tetracycline-susceptible isolates (data not shown). Our observations are consistent with previous reports showing that in *H. pylori* low-level tetracycline resistance is conferred by various single or double base pair substitutions at positions 926–928 and that high-level tetracycline resistance is conferred by the triple base pair substitution AGA926–928→TTC.¹¹ Low-level tetracycline resistance, especially when it is highly prevalent, should be taken into consideration when choosing an optimal second-line regimen for *H. pylori* eradication.

So far, most of the characterized *H. pylori* tetracycline-resistant isolates from different regions of the world, including those in this report, contain mutations in the same 16S rRNA domain that involves the primary tetracycline-binding site.^{4,16} Even so, tetracycline resistance among *H. pylori* isolates may also occur in the absence of mutations in the 16S rRNA gene.^{16,20,21} In this regard, a proton-motive force-dependent efflux mechanism has been suggested to play an important role in tetracycline resistance of *H. pylori* clinical isolates.²² Thus, complementary culture and susceptibility testing to assess *H. pylori* resistance are still necessary.

Besides confirming and extending an association between low-level tetracycline resistance in *H. pylori* with single or double base pair mutations affecting the primary tetracycline-binding site of the 16S rRNA, this study has shown that the PCR-RFLP/HinfI assay is not sufficiently reliable to be the sole method to detect low-level tetracycline resistance among Chilean *H. pylori* clinical isolates. Thus, whenever a 'tetracycline-susceptible phenotype' is obtained by PCR-RFLP/HinfI analysis of the 535 bp fragment, sequencing is required either to validate

the observation or to identify eventual single or double substitutions at nucleotides 926–928, which may sustain a low-level tetracycline-resistant phenotype. However, this molecular information must be complemented with well controlled tests for inhibition of growth at critical threshold levels of tetracycline, which are still the best measure of whether a strain is or is not tetracycline susceptible.

Acknowledgements

We are grateful to Drs C. Defilippi, C. C. Defilippi and A. M. Madrid (Gastroenterology Service, Clinical Hospital, University of Chile) for providing us with gastric biopsies. We also thank Mr L. Garrido and Mr C. Vallejos for their valuable technical assistance.

Funding

This research was partly supported by grants AMAYOR 02/4-2 from DID-University of Chile, Enlace ICBM-FM-UCh 2006 and Fondecyt 1085193 (H. T.).

Transparency declarations

None to declare.

References

- Dunn BE, Cohen H, Blaser MJ. *Helicobacter pylori*. *Clin Microbiol Rev* 1997; **10**: 720–41.
- Kuipers EJ. *Helicobacter pylori* and the risk and management of associated diseases: gastritis, ulcer disease, atrophic gastritis and gastric cancer. *Aliment Pharmacol Ther* 1997; **11** Suppl 1: 71–88.
- Romano M, Iovene MR, Russo MI *et al.* Failure of first-line eradication treatment significantly increases prevalence of antimicrobial-resistant *Helicobacter pylori* clinical isolates. *J Clin Pathol* 2008; **61**: 1112–5.
- Wueppenhors N, Stueger H-P, Kist M *et al.* Identification and molecular characterization of triple- and quadruple-resistant *Helicobacter pylori* clinical isolates in Germany. *J Antimicrob Chemother* 2009; **63**: 648–53.
- Megraud F, Marshall BJ. How to treat *Helicobacter pylori*. First-line, second-line, and future therapies. *Gastroenterol Clin North Am* 2000; **29**: 759–73.
- Gisbert JP, Pajares JM. *Helicobacter pylori* therapy: first-line options and rescue regimen. *Dig Dis* 2001; **19**: 134–43.
- Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 2001; **65**: 232–60.
- Mégraud F, Lehours P. *Helicobacter pylori* detection and antimicrobial susceptibility testing. *Clin Microbiol Rev* 2007; **20**: 280–322.
- Gerrits MM, van Vliet AHM, Kuipers EJ *et al.* *Helicobacter pylori* and antimicrobial resistance: molecular mechanisms and clinical implications. *Lancet Infect Dis* 2006; **6**: 699–709.
- Vallejos C, Garrido L, Cáceres D *et al.* Prevalence of metronidazole, claritromycin and tetracycline resistance in *Helicobacter pylori* isolated from Chilean patients. *Rev Med Chile* 2007; **135**: 287–93.
- Gerrits MM, de Zoete MR, Arents NL *et al.* 16S rRNA mutation-mediated tetracycline resistance in *Helicobacter pylori*. *Antimicrob Agents Chemother* 2002; **46**: 2996–3000.

- 12** Ribeiro ML, Gerrits MM, Benvenuto YHB. Detection of high-level resistance in clinical isolates of *Helicobacter pylori* using PCR-RFLP. *FEMS Immunol Med Microbiol* 2004; **40**: 57–61.
- 13** Garrido L, Toledo H. Novel genotypes in *Helicobacter pylori* involving domain V of the 23S rRNA gene. *Helicobacter* 2007; **12**: 505–9.
- 14** Tomb JF, White O, Kerlavage AR et al. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* 1997; **388**: 539–47.
- 15** Silva FM, Eisig JN, Chehter EZ et al. Omeprazole, furazolidone, and tetracycline: an eradication treatment for resistant *Helicobacter pylori* in Brazilian patients with peptic ulcer disease. *Rev Hosp Clin Fac Med Sao Paulo* 2002; **57**: 205–8.
- 16** Dailidienė D, Bertoli MT, Miciulevičienė J et al. Emergence of tetracycline resistance in *Helicobacter pylori*: multiple mutational changes in 16S ribosomal DNA and other genetic loci. *Antimicrob Agents Chemother* 2002; **46**: 3940–6.
- 17** Mendonça S, Ecclissato C, Sartori MS et al. Prevalence of *Helicobacter pylori* resistance to metronidazole, clarithromycin, amoxicillin, tetracycline, and furazolidone in Brazil. *Helicobacter* 2000; **5**: 79–83.
- 18** Glupczynski Y, Broutet N, Cantagrel A et al. Comparison of the E test and agar dilution method for antimicrobial susceptibility testing of *Helicobacter pylori*. *Eur J Clin Microbiol Infect Dis* 2002; **21**: 549–52.
- 19** Gerrits MM, Berning M, van Vliet AHM et al. Effects of 16S rRNA gene mutations on tetracycline resistance in *Helicobacter pylori*. *Antimicrob Agents Chemother* 2003; **47**: 2984–6.
- 20** Khan R, Nahar S, Mukhopadhyay AK et al. Isolation of tetracycline-resistant clinical *Helicobacter pylori* without mutations in 16S rRNA gene in Bangladesh. *Microbiol Immunol* 2008; **52**: 508–11.
- 21** Wu JY, Kim JJ, Reddy R et al. Tetracycline-resistant clinical *Helicobacter pylori* isolates with and without mutations in 16S rRNA-encoding genes. *Antimicrob Agents Chemother* 2005; **49**: 578–83.
- 22** Anoushiravani M, Falsafi T, Niknam V. Proton-motive force dependent efflux of tetracycline in clinical isolates of *Helicobacter pylori*. *J Med Microbiol* 2009; **58**: 1309–13.