The Laryngoscope

Prevalence of Clarithromycin-Resistant *Helicobacter pylori* in Patients With Chronic Tonsillitis by Allele-Specific Scorpion Real-Time Polymerase Chain Reaction Assay

Taghi Naserpour Farivar, PhD; Reza Najafipour, MD; Pouran Johari, BSc

Objectives/Hypothesis: To investigate the allelic prevalence of resistance to clarithromycin in the DNA of clinical isolates of *Helicobacter pylori* obtained from biopsy specimens of patients with chronic tonsillitis by Scorpion real-time polymerase chain reaction (PCR).

Study Design: Pathologic specimens of patients with chronic tonsillitis were used for rapid urease test, and blocks of paraffin-embedded tonsillar tissue were used for McMullen staining, rapid urease test, and Scorpion real-time PCR test.

Methods: A total of 103 biopsy samples were obtained from patients with chronic tonsillitis and examined for the presence of clarithromycin resistant *H. pylori*. Modified McMullen staining and rapid urease test were done on the all the samples. The DNA of specimens was extracted from the pathology blocks, and Scorpion real-time PCR was performed on a final volume of 25 μL.

Results: Of 103 biopsy specimens, 22 samples were identified as infected by *H. pylori*, of which none were sensitive to clarithromycin. One had the A2143G genotype, and four had the A2142G genotype. Two had a mixed sensitive and the A2143G genotype, and five had a mixed sensitive and A2142G genotype. One strain had a mixed genotype of sensitive, A2143G, and A2142G.

Conclusions: The reported rate of resistance to clarithromycin is of great variation among *H. pylori* strains isolated from specimens in different countries. Our study showed that the most prevalent genotypes in our *H. pylori*-positive specimens was A2142G followed by A2143G, which is different from reported results of allele-specific genotyping of *H. pylori* strains isolated from gastric biopsy and may be a result of cross-resistance to erythromycin and other macrolides.

Key Words: Scorpion real-time polymerase chain reaction, chronic tonsillitis, clarithromycin.

***Laryngoscope,* 123:1478–1482, 2013**

INTRODUCTION

It has been reported that the chronic pharyngitis might be associated with *Helicobacter pylori* infection.[1–5] Bacterial infections of the tonsils and adenoids are treated with various antibiotics, and surgical removal is considered in situations resistant to anti-*H. pylori* therapy or in frequently recurrent infections.[6]

So far, numerous studies regarding the determination of antibiotic resistance in clinical isolates of *H. pylori* have been carried out, leading to detection of antibiotic resistance among different strains of this bacterium worldwide.[7] The phenomenon of resistance to metronidazole, amoxicillin, tetracycline, as well as high-level resistance to clarithromycin have been emphasized in many previous studies.[8] Most studies to determine the drug resistance have been performed on *H. pylori* isolates obtained from adults, and there are little data regarding the determination of *H. pylori* antibiotic resistance in clinical isolates collected from children. Studies on detection of drug resistance of *H. pylori* in Iran have also been limited to a small amount of research carried out throughout the country.[7] Based on findings of numerous studies,[9] it is clear that the *H. pylori* resistance against clarithromycin, a key component of the triple drug treatment protocol to eradicate this organism, is considerably high, up to 20% in some countries. This level of drug resistance against clarithromycin is regarded as a major risk factor for treatment failure leading to a decrease of more than 70% in efficacy of the first-line drugs.[9–11]

Falsafi et al.,[7] in their study on the Iranian population, demonstrated the initial presence of a high level (25%) of resistance to clarithromycin among clinical isolates of *H. pylori*, whereas this antibiotic was unavailable in the country at the time of study.

The mechanism of resistance against clarithromycin by *H. pylori* is due to a decrease in attachment of this drug to the bacterial ribosome[12] that occurs through point mutations in the coding region of 23SrRNA for peptidyl transferase, leading to development of resistance against clarithromycin. There are three major point

From the From the Cell and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, Iran.

Editor's Note: This Manuscript was accepted for publication September 17, 2012.

This work was supported by a grant from Deputy of Research in Qazvin University of Medical Sciences.

The authors have no other funding, financial relationships, or conflicts of interest to disclose.

Send correspondence to Taghi Naserpour Farivar, MD, Associate Professor of Microbiology, Dean of the Cell and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin University of Medical Sciences, Qazvin, Iran. E-mail: tnaserpour@qums.ac.ir

DOI: 10.1002/lary.23777

mutations occurring in the vicinity of the 23SrRNA gene that are responsible for the development of resistance to clarithromycin in *H. pylori*.[12–14] The most common types of such mutations are A2142G and A2143G, and the most unusual one is the A2142C mutation.[15] Other rare mutations discovered so far include T2717C, A213G, G2141A, and T2182C mutations.[16–18] Because the A213G, G2141A, and T2117C mutations have been reported only once and there are no other reports of these mutations in the literature, and as the T2182C mutation is also seen in clarithromycin-sensitive isolates,[16] it seems that these mutations play little role in the development of resistance against clarithromycin.[19]

Several techniques of real-time polymerase chain reaction (PCR) for detection of susceptibility to clarithromycin have been described,[20,21] and among these the Scorpion real-time PCR is reported to have the potential to accurately differentiate between the diverse alleles of target nucleotides through a one-step process.[22–24]

Recently, it has been shown that there is clinically significant mutation information of isolated clarithromycin resistance of *H. pylori*. Hansomburana and colleagues reported that in their studied samples the most common mutation was A2142G, and it was associated with high MIC of clarithromycin.[25] Abadi and colleagues reported that high A2143G mutation frequency may increase treatment failure.[26]

Thus, considering the crucial importance of clarithromycin in treatment of *H. pylori* infections, the present study attempted to investigate the allelic mutation prevalence of resistance to clarithromycin in clinical isolates of *H. pylori* obtained from biopsy specimens of patients with chronic tonsillitis by Scorpion real-time PCR.

MATERIALS AND METHODS

This was an experimental study in which 103 biopsy samples were obtained from patients with chronic tonsillitis who visited the ear, nose, and throat clinic at Quds Hospital (affiliated with Qazvin University of Medical Sciences). These samples were examined for the presence of *H. pylori* and to determine the prevalence of resistance against clarithromycin. All patients had signs of chronic tonsillitis: sore throat, dysphagia,[27] and voice roughness[28] of more than 3-months duration. *H. pylori* presence was tested by serology, and the patients studied had no previous history of reflux or gastric disorders. Pathologic specimens of patients with chronic tonsillitis were used for rapid urease testing, and blocks of paraffin-embedded tonsillar tissue were used for McMullen staining and Scorpion real-time PCR testing.

Modified McMullen's Staining of Tonsillar Biopsy

The paraffin-embedded sections was dewaxed, dehydrated, and covered by carbolfuchsin for 2 minutes. The sections were rinsed and stained in malachite green for 2 minutes. The slides were then rinsed in tap water and air dried. Using this procedure, gull-shaped *H. pylori* were stained magenta against light green tonsillar tissue.

Rapid Urease Test

The rapid urease test (RUT) was performed using a commercial RUT kit obtained from Cham Enzyme (Tehran, Iran). For positive control, a standard strain of *H. pylori* ATCC 26695 was used, and distilled water was employed as a negative control.

The DNA of specimens was extracted from the pathology blocks after removal of paraffin from the prepared slice, and DNA extraction was carried out using a commercial extraction kit (Roche, Grenzach-Wyhlen, Germany).The DNA content of specimens was measured using the NanoDrop instrument (Applied Biosystems, Carlsbad, CA). The final concentration of DNA following application of the extraction procedure mentioned earlier was 200 ± 10 ng.

Scorpion Real-Time PCR

The primers used in this study were designed based on a protocol described by Burucoa et al.,[19] in which the target of amplification was the position of adenine next to the 23SrRNA gene. The forward primer, 23SF2, is a regular primer located 107 to 125 bases upstream of the adenine. The reverse Scorpion primers, which are complementary mutations to those that have to be detected, are designed to end their 3′ end in a manner used to design the 3′-mismatched reverse primers.[10,19] The primers used in the present study were similar to those used by Burucoa et al., except for replacing the quencher BHQ1 with BHQ2 within the 23SscA2142G oligonucleotide. Primers were purchased from Metabion Company (Martinsried, Germany). The Scorpion real-time PCR was performed on a final volume of 25 μL consisting of Premix Ex Taq (TaKaRa, Shiga, Japan), 5 μL of extracted DNA from the biopsy specimen or 1 μL of extracted DNA from culture, 0.1 μM of 23SF2 primer, 0.14 μM of Scorpion primer 23SscA2142G, 0.18 μM of 23SscA2143G, 0.1 μM of 23SscA2142C, and 0.08 μM of 23SscWT Scorpion primers.[7] Amplification was carried out according to Burucoa et al.,[19] and appropriate modifications were made for use in the ABI Prism 7500 Sequence Detection System (Applied Biosystems) as follows: denaturation step (95°C/3 seconds), followed by 50 cycles of reactions containing denaturation (95°C/3 seconds), annealing (55°C/34 seconds), and extension (72°C/20 seconds). A positive control of extraction was obtained by the detection of a fragment of a gene coding for β-hemoglobin by Scorpion real-time PCR using SYBR green detection canal and BGLO1 and BGLO2 primers.[19]

Scorpion real-time PCR positive control. Initially, PCR was performed on DNA of a standard strain of *H. pylori* ATCC 26695 and confirmed resistant strains,[29] followed by PCR on a mixture of wild types and mutants to re-examine the ability of this technique to detect *H. pylori*.

RESULTS

In our paraffin-embedded study samples, 19 samples had a positive modified McMullen's staining result, and from our 103 samples, 50 samples showed positive RUT. Of 103 biopsy specimens, 22 samples were identified as infected by *H. pylori* by Scorpion real-time PCR assay, of which nine were sensitive to clarithromycin (Fig. 1). One had the A2143G genotype, and four had the A2142G genotype. Two had a mixed sensitive and A2143G genotype (Fig. 2), and five had mixed sensitive and A2142G genotypes (Fig. 3). One strain had a mixed genotype of sensitive, A2143G, and A2142G. Distribution frequency of genotyped clarithromycin *H. pylori* strains are shown in Figure 4.

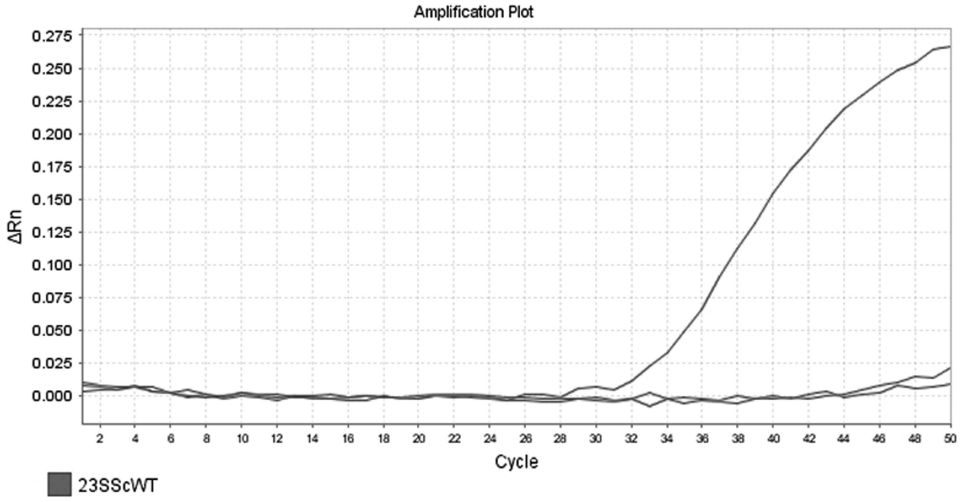

Fig. 1. Amplification plot of samples infected with sensitive *Helicobacter pylori* strains.

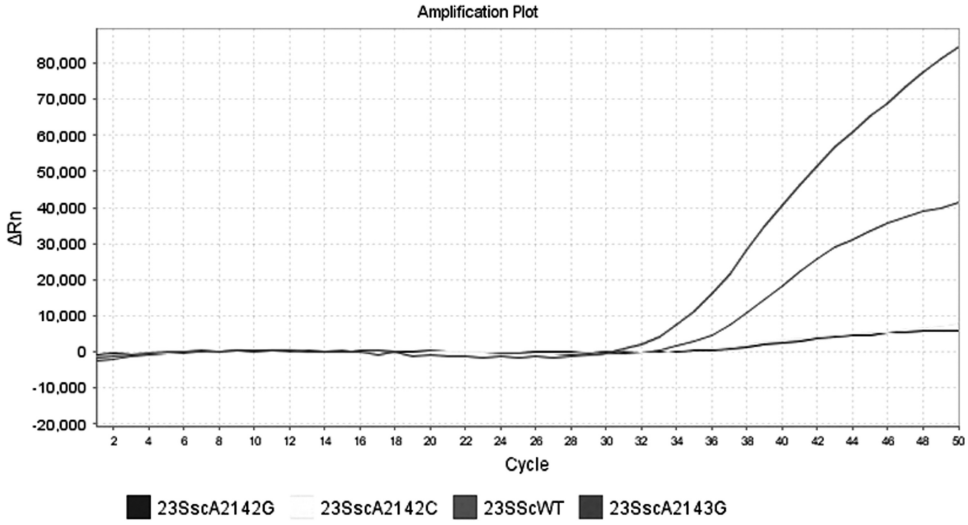

Fig. 2. Amplification plot of samples coinfected with sensitive and A2143G *Helicobacter pylori* strains.

Gel electrophoresis of Scorpion real-time PCR product is shown in Figure 5. A 140-bp amplicon is indicative of a successful amplification. Pathological diagnosis of 93 samples (90.3%) from our 103 studied samples was chronic tonsillitis, and 10 samples (9.7%) had a chronic tonsillitis plus follicular hypertrophy diagnosis. Our 103

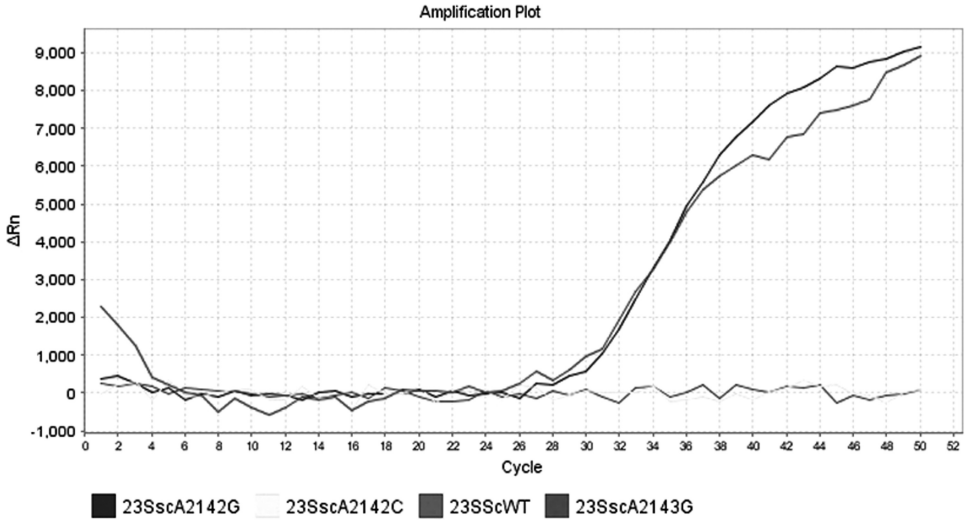

Fig. 3. Amplification plot of samples infected with sensitive and A2142G *Helicobacter pylori* strains.

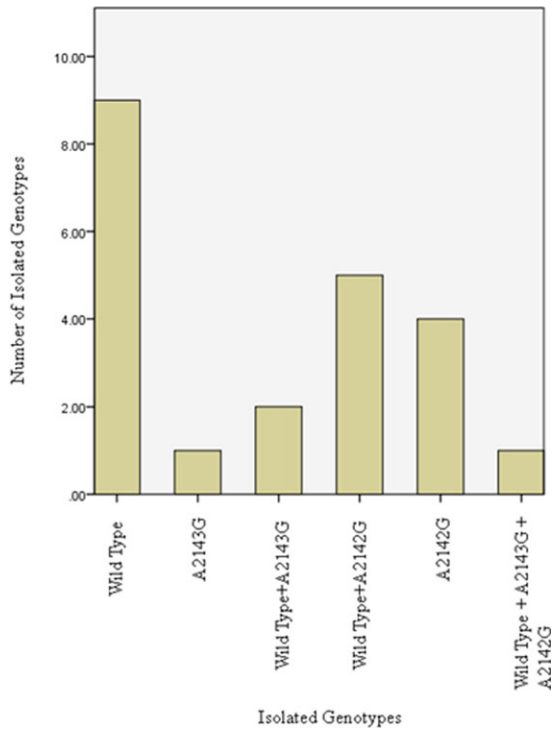

Fig. 4. Distribution of clarithromycin resistant genotypes among *Helicobacter pylori* isolated strains for gastric cancer patients. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

patients comprised 46 males and 57 females, with a sex ratio of 1:1.2, respectively. None of our patients were treated with clarithromycin before admittance for tonsillectomy. Age distribution of our study patients is shown in Table I.

TABLE I.
Age Distribution Among Studied Samples..

Age (yr)	No. of Samples
1–6.9	51 (49.5%)
7–14.9	41 (39.8%)
15–24.9	5 (4.9%)
≥25	6 (5.8%)

DISCUSSION

The reported rate of resistance to clarithromycin is of great variation among *H. pylori* strains isolated from gastric biopsy specimens in different countries, as shown in a study by Ahmad et al., where a resistant rate of 13.2% in *H. pylori* strains against clarithromycin was reported.[30] In some cases, the studies carried out in different parts of a single country are also indicative of considerable differences. In their study, Falsafi et al.[7] reported the existence of a resistance rate of as high as 25% against clarithromycin, whereas at the time of the study this drug was not in common use in the country yet. In another study by Megraud,[9] the rate of resistance to clarithromycin was reported to be around 20%.

In Iran, the study carried out by Falsafi et al.[7] indicated that the resistance rate against clarithromycin was 25%, whereas in another study by Fallahi and Maleknejad[31] on 24 cultures positive for *H. pylori* obtained from a total of 62 children, the resistance rate was reported to be approximately 16.4%. In other studies by Malekzadeh et al.[32] and Khashei et al.,[33] a resistance rate of 25.6% against clarithromycin was seen among the *H. pylori* strains obtained from gastric biopsy specimens from patients in Isfahan using both culture and randomly amplified polymorphic DNA-PCR.

In their study, Burucoa and colleagues[16] found that from 60 culture-positive *H. pylori* specimens, 24 were clarithromycin resistant, and of these samples 16 had A2143G, six had A2142G, and two had A2142G. Li and colleagues[34] found in their study that of 13 PCR-positive *H. pylori* samples, nine had wild-type genotypes, two had the A2143 genotype, and one had the A2142G genotype. These results do not confirm our results, in which of 103 specimens we had four A2143G mutants and one A2143G genotype.

Difference in the prevalence of A2192G genotypes in isolated bacteria in these studies may originate from different rates of cross-resistance to other macrolides. It has been reported that *H. pylori* strains that are resistant to erythromycin may be resistant to clarithromycin and vice versa.[35] Also, high resistance to erythromycin was observed in *H. pylori* strains isolated in Iran.[7,36]

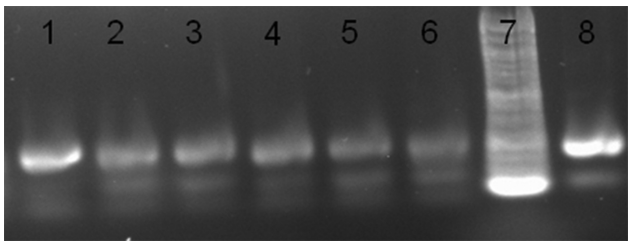

Fig. 5. Gel electrophoresis of Scorpion real-time PCR products. Lane 1: clarithromycin-sensitive *Helicobacter pylori*. Lanes 2–6: clarithromycin-resistant *H. pylori*. Lane 7: 50 bp DNA ladder. Lane 8: *H. pylori* American Type Culture Collection 26695. A 140-bp product is indicative of amplification.

CONCLUSION

Due to possible cross-resistance that may exist among macrolides in areas with a high incidence of erythromycin resistance, the evolutionary pattern of susceptibility to clarithromycin and other antibiotics must be accurately considered. On the other hand, significant coinfection of sensitive and clarithromycin-resistant *H. pylori* strains isolated in this study shows that this coinfection may be lost in the routine procedures of *H. pylori* drug sensitivity evaluation. Application of molecular methods along with routine antibiogram procedures are recommended.

BIBLIOGRAPHY

1. Zhang JP, Peng ZH, Zhang J, Zhang XH, Zhang XH, Zheng QY. Helicobacter pylori infection in the pharynx of patients with chronic pharyngitis detected with TDI-FP and modified Giemsa stain. *World J Gasteroenterol* 2006;12:468–472.

2. Lukes P, Astl J, Pavlik E, Potuznikova B, Sterzl I, Betka J. Helicobacter pylori in tonsillar and adenoid tissue and its possible role in oropharyngeal carcinogenesis. *Folia Biol (Praha)* 2008;54:33–39.
3. Vayisoglu Y, Ozcan C, Polat A, Delialioglu N, Gorur K. Does Helicobacter pylori play a role in the development of chronic adenotonsillitis? *Int J Pediatr Otorhinolaryngol* 2008;72:1497–1501.
4. Kurtaran H, Uyar ME, Kasapoglu B, et al. Role of Helicobacter pylori in pathogenesis of upper respiratory system diseases. *J Natl Med Assoc* 2008;100:1224–1230.
5. Ozcan C, Vayisoglu Y, Otag F, Polat A, Gorur K, Ismi O. Does Helicobacter pylori have a role in the development of chronic otitis media with effusion? A preliminary study. *J Otolaryngol Head Neck Surg* 2009;38: 526–531.
6. Najafipour R, Naserpour Farivar T, Pahlevan AA, Johari P, Safdarian F, Asefzadeh M. Agreement rate of rapid urease test, conventional PCR, and Scorpion real-time PCR in detecting Helicobacter pylori from tonsillar samples of patients with chronic tonsillitis. *J Global Infect Dis* 2012; 4:106–109.
7. Falsafi T, Mobasheri F, Nariman F, Najafi M. Susceptibilities to different antibiotics of Helicobacter pylori strains isolated from patients at the pediatric medical center of Tehran, Iran. *J Clin Microbiol* 2004;42: 387–389.
8. Fallahi GH, Maleknejad M. Helicobater pylori culture and antimicrobial resistance. *Indian J Pediatr* 1997;74:127–130.
9. Megraud F. Helicobacter pylori antibiotic resistance: prevalence, importance and advantages in testing. *Gut* 2004;53:1375–1384.
10. Dore MP, Leandero G, Realdi R, Sepulveda A, Graham YD. Effect of pretreatment antibiotic resistance to metronidazole and clarithromycin on outcome of Helicobacter pylori therapy: a meta-analytical approach. *Dig Dis Sci* 2000;45:68–76.
11. Houben MH, van de Beek D, Hensen EF, Craen AJ, Rauws EA, Tygat GN. A systematic review of Helicobacter pylori: mechanism and stability an strains from clarithromycin-treated patients. *Antimicrob Agents Chemother* 1999;41:2550–2553.
12. Occhialini A, Urdaci M, Doucet-Populaire F, Bebear CM, Lamouliatte H, Megraud F. Macrolide resistance in Helicobacter pylori: rapid detection of point mutations and assays of macrolide binding to ribosomes. *Antimicrob Agents Chemother* 1997;41:2724–2728.
13. Taylor DE, Ge Z, Purych D, Lo T, Hiratsuka K. Cloning and sequence analysis of two copies of a 23SrRNA gene from Helicobacter pylori and association of clarithromycin resistance with 23SrRNA mutations. *Antimicrob Agents Chemother* 1997;41:2621–2628.
14. Versalovic J, Shortridge D, Kibler K, et al. Mutations in 23SrRNA are associated with clarithromycin resistance in Helicobacter pylori. *Antimicrob Agents Chemother* 1996;40:477–480.
15. Van Doorn LJ, Glupzynski Y, Kusters JG, et al. Accurate prediction of macrolide resistance in Helicobacter pylori by a PCR line probe assay for detection of mutations in the 23SrRNA gene: multicenter validation study. *Antimicrob Agents Chemother* 2001;45:1500–1504.
16. Burucoa C, Landron C, Garnier M, Fauchere JL, Khan R, Rahman M. T2182C mutation is not associated with clarithromycin resistance in Helicobacter pylori [letter]. *Antimicrob Agents Chemother* 2005;49: 868–870.
17. Fontana C, Favaro M, Minelli S, Criscuolo AA, Pietroiusti A, Galante A, Favalli C. New site of modification of 23srRNA associated with clarithromycin resistance of Helicobacter pylori clinical isolates. *Antimicrob Agents Chemother* 2002;46:3765–3769.
18. Hulten K, Gibreel A, Skold O, Engstrand L. Macrolide resistance in Helicobacter pylori: mechanism and stability in strains from clarithromycin treated patients. *Antimicrob Agents Chemother* 1997;41:2550–2553.
19. Burucoa C, Garnier M, Silvaim C, Louis Fauchere J. Quadruplex real-time PCR assay using allele-specific scorpion primers for detection of mutations conferring clarithromycin resistance to helicobacter pylori. *J Clin Microbiol* 2008;46:2320–2326.
20. Matsumura M, Akiba YH, Ogura K, et al. Rapid detection of mutations in the 23SrRNA gene of Helicobacter pylori that confers resistance to clarithromycin treatment to the bacterium. *J Clin Microbiol* 2001;39: 691–695.
21. Schabereiter-Gurner C, Hirschl AM, Dragosics B, et al. Novel real time PCR assay for detection of Helicobacter pylori infection and simultaneous clarithromycin susceptibility testing of stool and biopsy specimens. *J Clin Microbiol* 2004;42:4512–4518.
22. Ng CT, Gilchrist CA, Lanb A, Roy S, Haque R, Houpt ER. Multiplex real time PCR assay using Scorpion probes and DNA capture for genotype specific detection of Giardia lamblia on fecal samples. *J Clin Microbiol* 2005;43:1256–1260.
23. Whitcombe D, Theaker J, Guy SP, Brown T, Little S. Detection of PCR products using self-probing amplicons and fluorescence. *Nat Biotechnol* 1999;17:804–807.
24. Xia QF, Xu SX, Wang DS, et al. Development of a novel quantitative real time assay using duplex scorpion primer for detection of Chlamydia trachomatis. *Exp Mol Pathol* 2007;83:119–124.
25. Hansomburana P, Anantapanpong S, Sirinthornpunya S, Chuengyong K, Rojborwonwittaya J. Prevalence of single nucleotide mutation in clarithromycin resistant gene of Helicobacter pylori: a 32-months prospective study by using hybridization real time polymerase chain reaction. *J Med Assoc Thai* 2012;95(suppl 3):S28–S35.
26. Abadi AT, Taghvaei T, Ghasemzadeh A, Mobarez AM. High frequency of A2143G mutation in clarithromycin-resistant Helicobacter pylori isolates recovered from dyspeptic patients in Iran. *Saudi J Gastroenterol* 2011;17:396–399.
27. Zautner AE. Adenotonsillar disease. *Recent Pat Inflamm Allergy Drug Discov* 2012;6:121–129.
28. Discolo CM, Darrow DH, Koltai PJ. Infectious indications for tonsillectomy. *Pediatr Clin North Am* 2003;50:445–458.
29. Siavoshi F, Safari F, Doratotaj JD, Khatami GHR, Falahi GHH. Antimicrobial resistance of Helicobacter pylori isolates from Iranian adults and children. *Arch Iranian Med* 2006;9:308–314.
30. Ahmad N, Zakaria WR, Abdullah SA, Mohamed R. Characterization of clarithromycin resistance in Malaysian isolates of Helicobacter pylori. *World J Gastroenterol* 2009;15:3161–3165.
31. Fallahi GH, Maleknejad S. Helicobacter pylori culture and antimicrobial resistance. *Indian J Pediatr* 2007;74:127–130.
32. Malekzadeh R, Mohammadnejad M, Siavoshi F, Massarraf S. Treatment of Helicobacter pylori infection in Iran: low efficacy of recommended western regimens. *Arch Iran Med* 2004;7:1–8.
33. Khashei R, Shojaei H, Adibi P. Genetic diversity and drug resistance of Helicobacter pylori strains in Isfahan, Iran. *Iranian J Basic Med Sci* 2008;11:174–182.
34. Li Y, Rimbaro E, Thirumurti S, Trespalacios A, Reddy R, Sabounchi S. Detection of clarithromycin resistance in Helicobacter pylori following noncryogenic storage of rapid urease tests for 30 days. *J Dig Dis* 2012; 13:54–59.
35. Xia HHX, Fan XG, Talley N. Clarithromycin resistance in Helicobacter pylori and its clinical relevance. *World J Gastroenterol* 1999;5:263–266.
36. Farshad S, Alborzi A, Japoni A, et al. Antimicrobial susceptibility of Helicobacter pylori strains isolated from patients in Shiraz, Southern Iran. *World J Gastroenterol* 2010;7:16:5746–5751.