Assessment of *Helicobacter Pylori* Prevalence by Scorpion Real-Time PCR in Chronic Tonsillitis Patients

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ABSTRACT

Background: Occasionally, bacteria or viruses enter the tonsils and these organs become overwhelmed by bacterial or viral infection leading to inflammation. Some studies confirmed the presence of *Helicobacter pylori* in tonsillar specimens of patients suffering from chronic tonsillitis and some others did not. The difference in results in various studies might be due to different laboratory methods. The aim of this study was to investigate the presence of *H. pylori* Deoxynucleic acid (DNA) in archival tonsillar tissues of patients with chronic tonsillitis by a rapid, sensitive, and specific technique of Scorpion real-time polymerase chain reaction (PCR). **Materials and Methods:** Scorpion real-time PCR and modified McMullen's staining was performed on 103 archival paraffin-embedded tonsillar samples collected from patients with chronic tonsillitis following tonsillectomy operation. **Results:** Our findings showed that *H Cell and Molecular Research Center. pylori* DNA was present in 21.35% of total specimens by using Scorpion real-time PCR. Modified McMullen's staining of paraffin-embedded sections was positive in 19 patients. Out of our 103 samples, 50 samples showed positive a rapid urease test whereas 53 samples demonstrated negative results, 20 produced positive PCR results, and 83 were negative for *H. pylori*. There was no significant relationship between the presence of *H. pylori*, sex, age, and place of residence. **Conclusion:** Although the existence of *H. pylori* in tonsillar tissue samples of patients with chronic tonsillitis is controversial, however, our results showed that in our studied specimens, a significant number of patients with chronic tonsillitis had *H. pylori* colonization.

Key words: *Helicobacter pylori*, PCR, Rapid urease test, Real-time, Scorpion, Tonsillitis

INTRODUCTION

Gastric mucosa extends to the upper aero-digestive tract mucosa and maintains its continuity. In several different studies, the colonization of *Helicobacter pylori* in locations beyond the gastrointestinal cavity such as adenotonsillar tissues[1,2] and nasal and sinus mucosa of patients[2,3] has been reported.

The diagnosis of chronic tonsillitis is mainly done by history and clinical examination[4] and these diagnostic criteria are not enough for diagnosis of *H. pylori* in related tonsillar clinical specimens and may lead to misdiagnosis of the illness. On the other hand, bacterial infections of the tonsils and adenoids are treated with various antibiotics and surgical removal is considered in situations resistant to medical therapy or in frequently recurrent infections. As *H. pylori* does not respond to routine antibacterial drugs that are used for chronic tonsillitis, the determination of the prevalence of *H. pylori* in tonsillar biopsy specimens of patients suffering from chronic tonsillitis may play a role in decision making in treatment, and with evaluation, the extent of the presence of *H. pylori* in these tonsillar specimens, and with proper antibiotic therapy against *H. pylori*, even some of the unnecessary tonsillectomy operations may be prevented.

There are studies in which the apparent existence of *H. pylori* in these tissue samples was confirmed[5-7] nevertheless, some other studies failed to show the presence of *H. pylori* in tonsillar materials examined.[8-11] The difference in results in various studies might be due to different laboratory methods.[6]

There are several different methods for the detection of

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H. pylori in clinical specimens; among those the culture of organisms, histopathlogy, polymerase chain reaction (PCR), and real-time PCR are the most widely used techniques. Real-time PCR is considered as the most rapid, sensitive, and accurate laboratory method for the detection of *H. pylori*.

Several real-time PCR assays for the identification of *H. pylori* have been designed. The Scorpion primer technology allows the precise discrimination between different alleles of a target nucleotide in a single-step process.[12-15] Using Scorpion primers, the sequence-specific priming and PCR product detection are achieved through the application of a single oligonucleotide.

The ability of the detection of the Scorpion real-time PCR assay not only for *H. pylori* but also for other infectious agents is the same as real-time PCR and is better than PCR-RFLP. On the other hand, the used probe in the Scorpion assay does not hydrolyze during the PCR process but in real-time PCR the probe hydrolyzes and so the efficacy of PCR in these methods is not the same.

Based on the reports of few studies with contradictory results,[1,2,5-10,12,13,16-19] this study was designed to detect the colonization of *H. pylori* in the biopsy specimens in paraffin-embedded tonsillar tissues of patients suffering from chronic tonsillitis.

MATERIALS AND METHODS

Patients and methods

This retrospective cross-sectional study was performed on 103 paraffin-embedded tonsillar specimens of patients who underwent adenotonsillectomy operation at the ENT ward of Ghods Hospital (Qazvin University of Medical Sciences, Qazvin, Iran) during 2010. All patients had common indications for adenotonsillectomy including recurrent tonsillitis, chronic tonsillitis, and/or adenoiditis. None of the patients had received any antibiotic or gastric acid-lowering drugs during the last 2 weeks prior to surgery. All parents and/or patients agreed with surgery and the study proposal.

Clinical samples

Initially, the paraffin-embedded tonsillar tissues were sectioned into 50-μm slices followed by DNA extraction using the DNA preparation protocol obtained from Section of Cancer Genomics, Genetics Branch, NCI, National Institutes of Health.

Modified McMullen's staining of tonsillar biopsy

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

Rapid urease test

The rapid urease test 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 employed as negative control.

Conventional PCR

DNA amplification was performed using the *H. pylori* detection kit (Cinnagen, Iran) in a final volume of 30 μl with the cycling program of the PCR instrument (Applied Biosystem, USA) consisting of a cycle of 72°C/30 s, 50°C/20 s, and 94°C/45 s followed by 30 cycles of 72°C/30 s, 50°C/20 s, and 94°C/20 s. Then 10 μl of the amplified sample was directly electrophoresed on 1.5% agarose gel. The presence of a 492-bp DNA fragment was indicative of a positive reaction.

Scorpion real-time

Scorpion real-time PCR was performed using ABI Prism 7500 Sequence Detection System (Applied Biosystem). The tails of the Scorpion primers were designed as previously described by Burucoa.[14] All primers and probes used were synthesized by Metabion(Germany; Table 1). The total volume of the real-time PCR was 20 μl containing 5 μl of DNA from a clinical sample or bacterial isolate, 0.1 μM of the oligonucleotide primer 23SF2, and 0.08 μM of 23SScWT, while the total volume of 20 μl was achieved by the addition of distilled water. The cycling conditions were adjusted in a way suitable to be used by ABI Prism 7500 Sequence Detection System (Applied Biosystem) with an initial denaturation at 95°C for 45 s, 50 cycles at 95°C for 15 s, 55°C for 34 s, and 72°C for 20 s. The acquisition of a signal was performed at 57°C during each cycle. A negative control for Scorpion real-time PCR was obtained by the observation of no amplification signal except internal amplification by adding DDW instead of DNA to the prepared real-time PCR master mix. A positive control for Scorpion PCR was constituted by using extracted DNA from *H. pylori* ATCC 26695. Positive extraction control was performed for each biopsy specimen in a separate tube in a final volume of 25 μl with Premix Ex Taq (TaKaRa,

Shiga, Japan), 5 µl of extracted DNA from the biopsy specimen, 0.25 µM of BGLO1 and BGLO2 primers, and ×0.5 Sybergreen 1 (Sigma-Aldrich,Germany). The cycling program was 1 cycle at 95°C for 10 s and 40 cycles at 95°C for 5 s, 55°C for 34 s, and 72°C for 10 s.

Statistical analysis

Data were analyzed using SPSS 11.5 software with 95% confidence intervals (95% CI). Also chi square and paired *t*-tests were used for statistical analysis.

RESULTS

In this survey, the prevalence of *H. pylori* infection in tonsils of patients with chronic tonsillitis was determined using the data obtained by the following procedures:

Modified McMullen's staining of tonsilar biopsy: Out of 103 samples from patients with clinical symptoms of chronic tonsillitis, 50 samples showed positive RUT whereas 53 samples demonstrated negative results.

Detection of *H. pylori* by rapid urease test: Out of 103 samples from patients with clinical symptoms of chronic tonsillitis, 50 samples showed positive RUT whereas 53 samples demonstrated negative results.

Detection of *H. pylori* by conventional PCR: When conventional PCR was used to detect *H. pylori* in biopsy specimens, of total 103 samples, 20 produced positive PCR results and 83 were negative for *H. pylori*. Since the present study was performed on paraffinized biopsies, there was no possibility of testing the samples by culture and recommended routine diagnostic tests.

Detection of *H. pylori* by Scorpion real-time PCR: Initially, Scorpion real-time PCR was performed on *H. pylori* ATCC 26695. Of total 103 samples, 21 biopsies were found to be positive for *H. pylori* by Scorpion real-time PCR whereas 82 samples showed negative results. Among samples with a positive Scorpion real-time PCR test, 17 samples were also RUT positive and 4 samples had RUT negative test results.

Scorpion real-time PCR on DNA extracts of *H. pylori* ATCC 26695 as control strains produced expected signals. The standard curve used to calculate the concentration of *H. pylori* DNA in unknown samples is presented in Figure 1a. The linear regression coefficient was 0.996 and the efficiency of PCR 99%. Figure 1b shows the fluorescent curves of the standard serial dilution from 1×10^5 to 1×10^2. The electrophoresis pattern of Scorpion real-time PCR products in agarose gel is shown in Figure 2. A 140 bp fragment in the peptidyl transferase gene of 23S rRNA showed a successful amplification. Of the total patients (103 cases) with tonsillitis who entered the study, 22 cases (21.35%) were found to have *H. pylori* DNA in their tonsillectomy specimens by the Scorpion real-time PCR method. Our study showed that there is no significant association between the sex, age, marital status, history of gastric involvement, gastric disease, and the presence of bacterium. Also no relationship was established between the sex, family income, and accommodation variables and the colonization of *H. pylori* in tonsil tissues (data not shown).

DISCUSSION

There are many arguments about different aspects of the association between *H. pylori* and chronic tonsillitis. Kusano and colleagues[15] in their recent study reported that the tonsillar *H. pylori* is unable to grow in any known culture media; however, Pavlic *et al*.[7] in their publication claimed

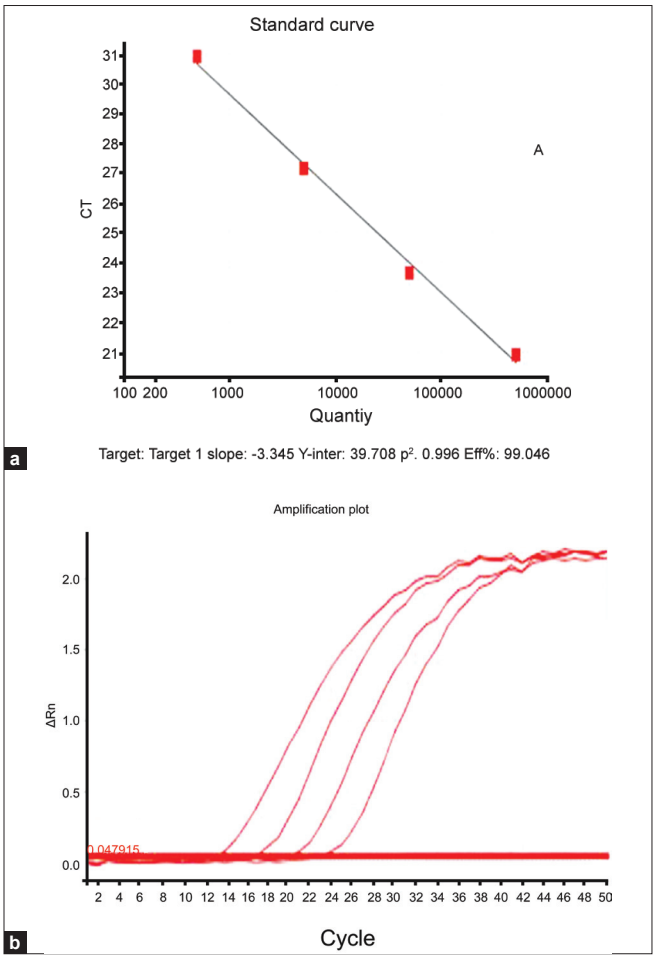

Figure 1: (a) Standard curve used to calculate the concentration of *H. pylori* DNA in unknown samples. The linear regression coefficient is 0.996 and the efficiency of PCR is 99%; (b) fluorescent curves of the standard dilution series. From the left to the right, 10^5, 10^4, 10^3, 10^2, and the negative control is presented by the horizontal curve

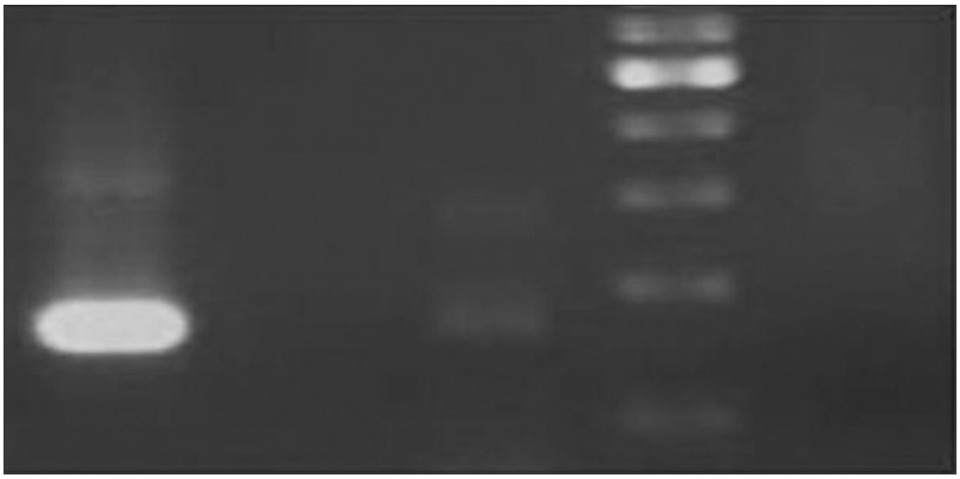

Figure 2: The PCR product was electrophoresed by agarose gel electrophoresis and stained with ethidium bromide. Lane 1: *Helicobacter pylori* ATCC 26695 as positive control; lane 2: negative control; lane 3: patient's positive sample; lane 4: 100 bp DNA ladder

of successful culture of *H. pylori* obtained from chronic tonsillitis and tonsillar cancer specimens.

This controversial issue is also present when it comes to the application of a molecular technique such as PCR. Eygor *et al*.[12] and Di Bonaventura *et al*.[10] reported of their failure in detecting *H. pylori* in tonsillar samples of patients by PCR (UreC and Urec2 primers) but Pavlic *et al*.[7] and Bulut *et al*.[13] both reported of the applicability of PCR in the detection of *H. pylori* in tonsillar specimens of their patients.

Zahedi *et al*. showed that 70 patients (73.7%) of their total 95 patients who underwent tonsillectomy had a positive anti-*H. pylori* IgG antibody in their sera, and the results of the rapid urease test on adenotonsil samples indicated that 42.1% of the specimens were positive for *H. pylori*.[6] Hence, it was concluded that the tonsils and adenoid tissues are the candidate places for the growth of *H. pylori*. In another study by Jabbari *et al*., of total 285 patients, 39.6% were positive for *H. pylori* in histopathologic examination, with 40 patients with positive RUT and 15 patients with a positive serum IgG anti-*H. pylori* level. In 40 patients, the results for both histopathology and RUT were positive although in 172 patients the findings associated with histopathology and RUT were negative and they deduced that *H. pylori* is present in the tonsillar tissue but the RUT is insensitive for the diagnosis of *H. pylori* in the tonsillar tissue, indicating that the tonsillar tissue might have a role in being a reservoir of *H. pylori* in children.[9]

Consistent with results mentioned earlier, Minocha *et al.,*[5] reported of reduced colonization of *H. pylori* infection in years following surgical adenotonsillectomy and decreased risk of *H. pylori* infection in gastric mucosa leading to hypothesize that the tonsil is a reservoir for *H. pylori*. In two studies by Jelavic *et al*.[8] and Skinner *et al*.,[13] it was shown that the tonsils are not an important reservoir for *H. pylori* transmission in children; however, Unver *et al*.[1] reported of a different finding indicating the presence of *H. pylori* positivity in patients who underwent adenotonsillectomy. In a study by Bulut *et al*.,[20] a relationship between *H. pylori* infection and adenotonsillar hypertrophy was found. Likewise, Cirak *et al*.[2] reported of the presence of cagA in *H. pylori*-positive tonsil and adenoid tissues.

In our study, Scorpion real-time PCR was positive in 21.35% of cases, a finding consistent with the results reported by Unver *et al*.,[1] Cirak *et al*.,[2] Minocha *et al*.,[5] Zahedi *et al*.,[6] Pavlic *et al*.,[7] and Bulut *et al*.,[20] yet in disagreement with findings of several other studies described by Jelvavic *et al*.,[8] Jabbari *et al*.,[9] Di Bonaventura *et al*.,[10] Eygor *et al*.,[12] Skinner *et al*.[13]

The differences in results of various studies might be due to diverse laboratory methods;[21,22] however, the high sensitivity and specificity of Scorpion real-time PCR compared to other laboratory techniques, in particular when combined with the application of 23S rRNA of bacterial genome as the target for amplification, makes the authors of the present research more confident over the results obtained through Scorpion real-time PCR testing of tonsillar tissues samples.

CONCLUSION

Our study showed that a significant number of patients with chronic tonsillitis may have *H. pylori* colonization. On the other hand, according to findings of numerous studies, it is clear that the *H. pylori* resistance against clarithromycin, a key component of triple drug treatment protocol to

eradicate this organism, is considerably high. This drug resistance against clarithromycin is regarded as the major risk factor for treatment failure leading to a decrease of higher than 70% in the efficacy of the first-line drugs. So, the modification of the antibiotic regime against *H. pylori* might be considered.

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