Comparative Analysis of Helicobacter Pylori Genotype in Natural and Drinking Waters of Isparta and its Surroundings with Gastric Mucosa of H. Pylori Infected Patients

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ABSTRACT

Objective: *There are studies that conclude that Helicobacter pylori is transmitted through fecal–oral and oral–oral routes. In this study, we aimed to determine the source and mode of transmission of H. pylori in H. pylori infected patients by comparing H. pylori species isolated both from drinking water samples and gastric mucosa samples obtained by endoscopy from people who had dyspeptic symptoms and had been using these sampled water supplies as drinking water by biochemical and genotypic analysis.*

Material and Method: *Twelve drinking water samples from different points and 37 gastric mucosa samples obtained by endoscopy from patients were collected. 109 bp length DNA segments from 16S rRNA location of H. pylori genome were amplified by nested PCR method from all water and human samples and the amplified genome were sequenced obtained from 7 of 12 water samples and 27 of 37 biopsy samples. The sequences were compared with the data from NIH by BLAST search program. Alignment has been performed by MEGA 4 alignment program between water and patient samples.*

Results: *All the DNA sequences were detected to be related to the H. pylori genome with 92 to 98% similarity. With alignment, 85% to 100% matching were detected between patient and water supply samples of which all were already confirmed as H. pylori genome (92 to 98% similarity).*

Conclusion: *These results suggest that contaminated and insufficiently disinfected drinking water supplies might be a reservoir for H. pylori which is a readily source for transmission to human in our region.*

Key Words: *Helicobacter pylori, 16S rRNA, sequence, drinking water, gastritis, transmission.* ***Nobel Med 2011; 7(2): 9-14***

ISPARTA VE ÇEVRESİNDE GASTRİK MUKOZALARI H. PYLORI İLE ENFEKTE OLAN HASTALARDA HELICOBACTER PYLORI GENOTİPİNİN YER ALTI VE İÇME SULARINDA KARŞILAŞTIRMALI ANALİZİ

ÖZET

Amaç: Bulaş yolu tam olarak bilinmemekle beraber, *H. pylori*'nin fekal-oral yolla bulaştığına ilişkin bir çok çalışma bulunmaktadır. Bu çalışmada, doğal ve içme suyu kaynağından su numunesi ve eş zamanlı olarak bu su istasyonlarındaki suyu içme suyu olarak kullanan ve dispeptik yakınmalar nedeniyle endoskopi yapılan hastalardan mide mukozası biyopsi örnekleri alınmış ve biyokimyasal ve genotipik testleri yapılarak incelenmiştir.

Materyal ve Metod: Oniki su istasyonunun 7'sinden alınan su numunelerinde ve çalışmaya alınan 37 hastanın 27'sinin mide mukozasında nested PCR yöntemi ile 109 bp uzunluğundaki bölgenin 16S rRNA tekniği ile amplifikasyonu yapılmıştır. İzole edilerek PCR da amplifikasyonu sağlanan bölgelerin sekansları BLAST Search programında var olan NIH data bankasındaki DNA dizilimleri ile karşılaştırılmıştır. Su numuneleri ve hastalardan alınan örneklerdeki dizi hizalamaları MEGA 4 Hizalama programı kullanılarak yapılmıştır.

Bulgular: DNA sekansı elde edilen tüm numunelerin %92-%98 benzerlikle *H. pylori* olduğu saptanmıştır. Yüzde 92-100 benzerlik oranlarıyla *H. pylori* genomu olarak teyid edilmiş hasta ve içme suyu numunelerinin hizalanması ile %85-100 arasında aynılık saptanmıştır.

Sonuç: Bu bulgular, *H. pylori*'nin sulardan bulaşması ve yayılmasında kontamine olmuş ve yetersiz arıtılmış içme sularının kaynak oluşturabileceği gösterilmiştir.

Anahtar Kelimeler: *Helicobacter pylori*, 16S rRNA, sekans, içme suyu, gastrit, bulaş **Nobel Med 2011; 7(2): 9-14**

INTRODUCTION

Helicobacter pylori is a widely recognized bacterium that can lead to several gastrointestinal diseases, such as gastritis, gastro duodenal ulcer, gastric carcinomas, and mucosa-associated lymphoid tissue lymphoma.[1] It has been estimated that more than half of the world's population is infected with this organism.[2] The prevalence rates of *H. pylori* infection appears to be different in developed and developing countries.[3,4] In developing countries, infection occurs early in life with a reported prevalence 50% in children, while in developed countries the prevalence of infection in children is usually 10%.[5] Despite such a high prevalence of infection, the reservoir of the bacteria and mode of transmission remains undetermined. Fecal-oral and person-to person contact have been suggested as possible routes of exposure to this organism.[2,6,7] Survival of this organism in distilled water, saline water, and artificial seawater has been reported to extend from days to weeks at temperatures between 4 and 15^{0}C and over a wide pH range.[8-10] *H. pylori* can resist to disinfection practices normally used in drinking water treatment in the viable but nonculturable form (VBNC), which will allow them to reach final consumption points.[11] However, this transmission mode is not easy to demonstrate because *H. pylori* is difficult to be recovered from aquatic environments.[12] In this sense, VBNC state of *H. pylori* can be a problem of great public health concern, since this form of the bacterium can not be detected by traditional culture methods. Attempts to culture *H. pylori* from environmental water samples have been mostly unsuccessful.[13,14] Unsuccessful attempts to culture *H. pylori* from environmental waters have led to the use of molecular methods to detect and identify this form. The presence of *H. pylori* DNA in river water, well water, waste water and surface and shallow groundwater has been detected by molecular methods.[2,15,16] The presence of *H. pylori* in source or finished water has profound public health and epidemiological implications. In addition, the potential presence of the organism in source water necessitates the documentation of treatment and disinfection procedures to prevent infection of drinking water consumers.

The aim of this study was to determine the source of *H. pylori* in *H. pylori* infected patients by comparing *H. pylori* species isolated both from drinking water and gastric mucosa samples obtained by endoscopy from people who had dyspeptic symptoms and had been using these sampled water supplies as drinking water by biochemical and genotypic analysis. We confirmed all the sequenced DNA products as *H. pylori* genome from NIH by BLAST program and compared the genetic similarity of DNA between water sources and biopsies by the MEGA 4 alignment program.

MATERIAL and METHOD

Gastric isolates

The study was carried out in patients who have dyspeptic symptoms and using sampled water →

supplies as a drinking water. A total of 37 patients referred to endoscopy with dyspeptic symptoms lasting longer than 3 months were enrolled in the study. Patients who

received proton pump inhibitors, histamine-2 receptor antagonists or bismuth containing compounds in the preceding month, and non-steroidal anti-inflammatory drugs in the preceding 15 days before endoscopy, patients with abnormal bleeding tests, allergic to anesthetics, pregnant patients, and patients who have had gastroduodenal surgery were excluded.

Informed consents were obtained from the eligible patients after the nature of the procedure was explained. The study was performed in compliance with human-studies guidelines and the study has been approved by the local ethical committee of Medical School. During the esophago-gastro-duodenoscopy, four biopsy specimens were sampled of which two from gastric antrum and two from corpus and frozen at -80°C until analysis.

Water samples

Water samples were collected from 12 different points at five different locations including natural surface waters and municipal water system in Isparta and its surrounding area under sterile conditions and transported in ice to the laboratory for analysis. Two liters of water were collected and centrifuged at 11,000 rpm for 30 min to final volume of 2 ml and stored at -80^{0}C for DNA extraction. Water samples were also analyzed for enumeration of total aerobic counts, total coliforms, and fecal coliforms.

DNA extraction

The biopsy samples were homogenized by sterile lancet in sterile petri dishes before defrosting. Genomic DNA's of both water and biopsy samples were extracted by using the QIAmp DNA Stool Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions.

PCR amplifications

H. pylori amplification was performed by nested PCR using 2 sets of primers that amplify 109 bp region of 16s rRNA gene locus. Three oligonucleotide primers were used with sequences (expressed 5' to 3') as follows: Hp1, CTG GAG AGA CTA AGC CCT CC (position 834 to 853); Hp2, ATT ACT GAC GCT GAT TGT GC (position 744 to 763); and Hp3, AGG ATG AAG GTT TAA GGA TT (position 407 to 426) as described in Adiloglu et al. [17] The *H. pylori* ATCC 43629 strain was used as a positive control and reaction mixture without DNA was used as a negative control for the whole procedure. The first amplification was performed with the Hp1 and Hp3 primers in 50 µl reaction mixture containing 50 mMKCl, 10 mM Tris-HCl (pH:8.3) 2.5 $mMMgCl_2$ solution, 200 µM dNTP's of dATP, dCTP, dGTP and dTTP each, and 1 unit Taq DNA polymerase enzyme (Promega Corporation, Madison WI, USA). After amplification as described below, 1 µl from one to ten dilution of the primary amplification product was used in a 50 µl reaction mixture with primers Hp1 and Hp2 under the same conditions described above. Amplification was performed by BioRad MJR PTC 100 "thermalcycler" (BioRad, USA).

In the first PCR, DNA was amplified for 29 cycles consisting of 20 s at 94^{0}C, 40 s at 55^{0}C, and 1 min at 72^{0}C, followed by a final extension step of 5 min at 72^{0}C. In the second PCR, DNA was amplified for 29 cycles consisting of 20 s at 94^{0}C, 30 s at 55^{0}C, and 40 s at 72^{0}C. The elongation step was at 72^{0}C for 5 min. The products were electrophorized on a 2% agarose gel and viewed by ethidium bromide staining. Positive and negative controls were included in all assays to monitor specificity and laboratory contamination during the analyses. Samples were interpreted as being positive for the presence of Helicobacter DNA if the assays produced a fragment comparable in size to that of the positive control and on the same line with 109 bp fragment of DNA ladder.

Automated DNA sequencing from 109 bp strands of positive samples were performed using standard protocols in ABI PRISM 310 DNA Sequencer (AME Bioscience, Norway). DNA sequences were assessed to their similarities to published DNA sequences using the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST). DNA sequences that are obtained from the water sources and the patient gastric biopsies were also aligned using MEGA 4 program (Biodesign Institute, AZ, USA).

Morphological and biochemical identification

Cultures and identification of *H. pylori* were performed immediately after the biopsies reached the laboratory. Biopsies were inoculated on to Colombia Agar, supplemented by *Helicobacter* selective supplement, Chocolate Agar and *H. pylori* selective agar after they are chopped by lancet in sterile conditions. The inoculated media were incubated for 3 to 7 days at 37^{0}C in BBL™ CampyPak Plus™ Microaerophilic System to provide microaerophilic environment (BD Diagnostic Systems, NJ, USA). Incubation was completed to 7 days unless visible colonies were present on the plates controlled on third and proceeding →

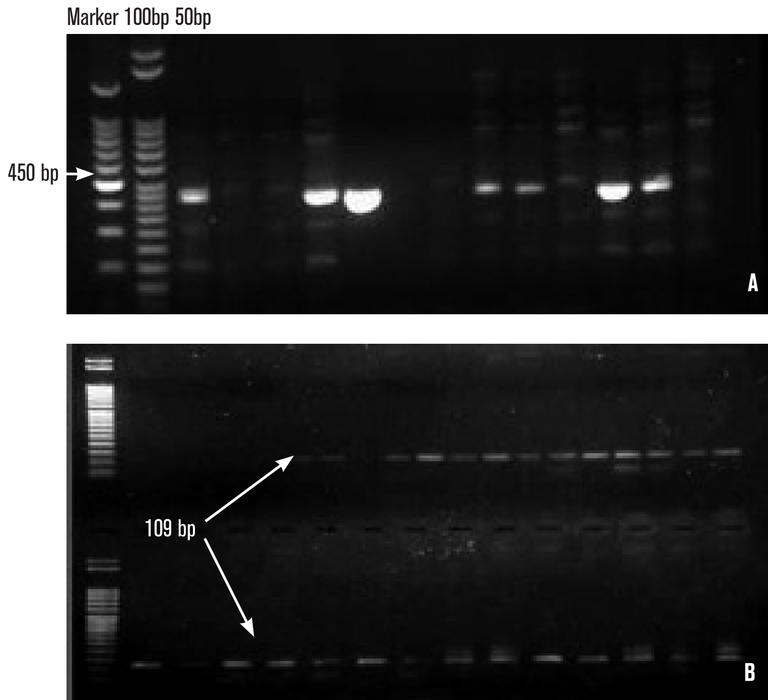

Figure 1. PCR amplification of DNAs from biopsy samples. (**A**) PCR I (**B**) PCR II. Agarose gel electrophoresis stained with ethidium bromide of 16S RNA H. pylori PCR products isolated from biopsy samples. First lanes are DNA molecular weight marker (Sigma-Aldrich, St. Louis, MO).

days every day up to seventh day. Translucent, water-drop like, convex, S type colonies were considered as possible *H. pylori* colonies and further evaluated for identification. Gram-negative comma like bacilli and catalase, urease and oxidase positive bacteria were further confirmed phenotypically as *H. pylori* with API Campy Identification Kit (BioMerieux, Pasteur Merieux, France).

RESULTS

Among the biopsy specimens sampled from gastric antrum and corpus of 37 patients, only four of the samples were able to be grown in culture on *H. pylori* selective agar. There were band formations in screened gels after DNA isolation from all samples taken from patients including the non culturable isolates. None of the water samples were able to be detected in cultures.

In the second PCR, PCR analysis of the biopsy samples yielded 27 bands out of 37 samples with size of 109 bp. PCR analysis of water samples yielded 7 bands out of 12 samples with possible size of 109 bp. Some *H. pylori* PCR products isolated from biopsy and water samples are demonstrated in Figures1 and 2, respectively.

The amplified genome was sequenced obtained from 7 of 12 water samples and 27 of 37 biopsy samples. Sequencing was successfully performed from all the amplified DNA fragments and the sequences were compared with the data from NIH by BLAST search program and all the samples were found to be related to the *H. pylori* genome with 92 to 98% similarity. Alignment has been performed by the MEGA 4 alignment program between water and patient samples who had consumed the sampled water supplies. Eighty five to 100% matching was detected between patient and water supply samples of which all were already confirmed as *H. pylori* genome (92 to 98% similarity) by the BLAST program.

DISCUSSION

The results were similar with many studies. [18,19] Although culturability has been the focus of many investigations of *H. pylori*, none has examined culturability in a potential natural reservoir, such as a freshwater environment. [2,15,16,18] The reason might be that, the exposure to the environment induces this organism to enter the VBNC state and the organism persists in the environment until it enters a suitable host. [14]

Nested PCR reactions were carried out in both biopsy and water samples. The expected amplified product size in PCR I was 450 bp, and 109 bp in PCR II. [17] As shown in Figure 1A, band formation was screened only in 8 patients out of 37 patients' gastric antrum and corpus after PCR I. However, there were band formations in 26 samples of gastric antrum and corpus after PCR II (Figure 1b) with the possible size of 109 bp.

PCR analysis of water samples yielded 7 bands out of 12 samples with possible size of 109 bp. Among the water samples which had band formations, two of them were from the main surface water source of the surrounding area. They are open to contamination by treated and untreated industrial and household sewage, and transported through canals to the main drinking water treatment plant, where it is treated by cascade aeration with chlorination; coagulation by using alum (if source water has high level of turbidity); flocculation (if coagulation process use); and sand filtration. Residual chlorine in the distribution system is adjusted approximately to 0.3-0.8 mg/L to prevent the bacterial recontamination.

Three of the water samples which were *H.pylori* positive were collected from tap water obtained mainly from the central drinking water treatment plant but there was also some ground water mix with an unknown amount. Baker et al. [20] indicated that if *H. pylori* gains access into the distribution system, via from either a break in treatment or infiltration into the system itself, it may be able to survive within the distribution system, where the level of oxidizing →

disinfectant is reduced. Geldreich [21] reported a free chlorine concentration of 0.1 to 0.3 ppm as typical of distribution systems. This concentration is well within the range in which *H. pylori* is more resistant to free chlorine than *E. coli*. Therefore, *H. pylori* might persist in a drinking water distribution system even though *E. coli* may not be detected simultaneously.

Especially, the water collected from public drinking fountain located in the center of the city and used by all local people and visitors was *H. pylori* positive. This fountain is 100% underground water and has high amount of flour. Since most of the people living in this area drink from this fountain, and the restaurants located in the centre of the city still use this water, it kind of represents the water source of the area. The study of Baker et al. [20] resulted that the presence of *H. pylori* in private drinking water supplies was associated with the presence of *E. coli*, a traditional indicator of fecal pollution. Similarly, all the samples which were *H. pylori* positive were also *E. coli* positive (Data not shown).

The amplified target DNAs were compared by the sequences from the data bank with the BLAST search program. The patient biopsy samples and the water sources showed 92-98% similarity to *H. pylori* DNA. The obtained DNA's from 7 different water sources and the patient samples were aligned almost perfectly to each other especially from the water source Karbuz drinking fountain (100%). These data demonstrated that the water source used by the patients as drinking water was the source of *H. pylori* which was isolated from the patients biopsies.

This is the first study comparing *H. pylori* species isolated both from drinking water samples and gastric mucosa samples obtained by endoscopy from people who had dyspeptic symptoms and had been using these sampled water supplies as drinking water by genotypic analysis.

Watson et al. [22] proved that 3 out of 6 sequences obtained from the water distribution system were *H. pylori* DNA. They also amplified 109 bp fragments of *H. pylori* with Hpl-Hp2 primers by nested PCR method. Lu et al. [19] sequenced 16S rRNA of 27 *H. pylori* isolated from wastewater samples. They sequenced 520 bp of this locus. The PCR detection rates for *H. pylori* also varied according to the assays. [22] In a Swedish study, [23] the detection rates were 40% for an adhesion gene assay compared with 12% for the 16S rRNA gene assay. Rates of 50 and 23% were reported for those two particular assays in a Mexican study [2] while a similar overall rate of 42% by the 16S rRNA gene assay was found in Mexico. [7]

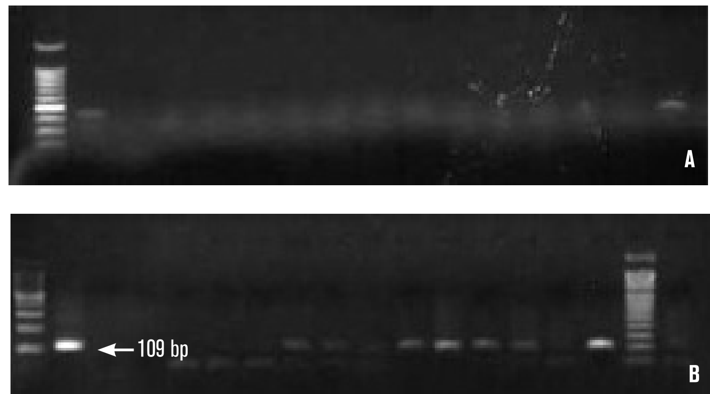

Figure 2. PCR amplification of DNAs from water samples. (A) PCR I (B) PCR II. Agarose gel electrophoresis stained with ethidium bromide of 16S RNA H. pylori PCR products isolated from water samples. Lanes 1 and 16, DNA molecular weight marker (Sigma-Aldrich, St. Louis, MO).

The results of Ho et al. [24] showed that PCR amplification and partial 16S rRNA gene sequence analysis strongly supported the contention that the genomes previously recovered from a pig, baboon, and rhesus monkeys gastric tissues were *H. pylori* DNA. The isolated DNAs from these animals' gastric tissues were amplified with *H pylori* specific primers and yielded PCR products identical to those from human isolates of *H. pylori* as confirmed by the use of a 20 based radio labeled probe complementary to an internal sequence flanked by the *H. pylori* specific primers.

In a study conducted in Japan, tap, river, sea and well waters were screened for *H. pylori* by adhesion ureA and 16S rRNA gene locuses. From these samples, only 2 of the 6 well samples contained 16S rRNA gene segment and some of the users of the well had *H. pylori* infection in the past. So they concluded that water borne transmission via well water may occur even in towns in Japan. [25]

Reinfection of *H. pylori* after eradication of the bacteria is a common problem in developing countries. A meta-analysis revealed an annual recurrence rate of 1.45% in the developed and 12% in the developing countries. [26] In this study, it is concluded that infected drinking water may be most important cause of reinfection.

The present data demonstrated that *H. pylori* is present in drinking water consumed in Isparta and consumption of such untreated drinking water is strongly associated with gastric colonization by the same bacteria confirmed by genotyping. This research also proves that oral transmission is one of the routes of transmission of pathogenic *H. pylori* to the patients. Current research will provide essential information concerning the route of transmission and epidemiology of *H. pylori*. Such information →

will allow public health officials, epidemiologists and risk assessors to objectively estimate the risk posed to individuals via consumption of drinking water.

In addition, this research will provide essential information for the control of spreading of this organism and the assurance of a safe drinking water supply.

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