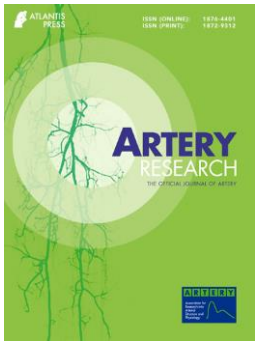

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Absence of *Helicobacter pylori* infection in coronary atherosclerosis disease in Northeast of Iran

Mohammad Hadi Sadeghian [a], Seyed Abbas Tabatabaee Yazdi [a], Hossein Ayatollahi [a,*], Kiarash Ghazvini [b], Mohammad Reza Keramati [a], Ehsan Ghayoor Karimiani [c], Maryam Sheikhi [c], Somaye Vakili Ahrarirodi [d], Ghohar Shaghayegh [c]

[a] *Cancer Molecular Pathology Research Centre, Ghaem Medical Centre, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*
[b] *Department of Microbiology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*
[c] *Cancer Molecular Pathology Research Centre, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*
[d] *Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*

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Abstract *Background:* Atherosclerosis is an inflammatory disorder in which infectious agents are incriminated as contributors. It is suggested that *Helicobacter pylori* may be a risk factor of atherosclerosis.
Methods: This study was performed whether there are differences between coronary artery with and without atherosclerotic plaque in the presences of *H. pylori* DNA using polymerase chain reaction (PCR) method.

This case-control study carried out on formalin-fixed paraffin-embedded (FFPE) of coronary artery biopsies. These specimens were obtained from 30 patients with coronary atherosclerosis and 30 subjects without atherosclerosis. Multiple sections were cut from each FFPE, the Deoxyribonucleic acid (DNA) was extracted by non-heating extraction method, then PCR was carried out for detection of *H. pylori* DNA and finally its products were analyzed by electrophoresis.
Results: The age range in the patient's group was from 18 to 50 years and the male to female ratio was 5 to 1. None of the coronary tissue samples had positive PCR results for *H. pylori* DNA

* Corresponding author. Tel.: +98 5118012584.
E-mail address: ayatollahih@mums.ac.ir (H. Ayatollahi).

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and all of the control samples were also negative.
Conclusions: This study showed that *H. pylori* infection is not associated with coronary artery atherosclerosis.

Introduction

Coronary artery disease (CAD) is one of the most common causes of mortality and morbidity in the world and obstructive atherosclerotic lesions are the main cause of CAD. It is well documented that atherosclerosis is an inflammatory process of large- and medium-sized arteries throughout the cardiovascular system which starts in childhood. Hypercholesterolemia, diabetes mellitus, high blood pressure and smoking are identified as the major risk factors for the development of atherosclerosis. In addition, the older age, male sex, family history and genetic factors predispose patients to coronary artery disease.[1]

Infections have long been suggested to play a critical role in the etiology of arteriosclerosis. Although there were some studies that have postulated the potential role of several infectious agents in exacerbation of inflammation in the pathogenesis of atherosclerosis, the role of infections in the pathogenesis of atherosclerosis is still a matter of debate. The relationship between atherosclerosis and some infections such as *Chlamydia pneumoniae*,[2] *hepatitis C virus*,[3] *Human immunodeficiency virus*,[3] *hepatitis B virus*,[4] *cytomegalovirus*,[5] *herpes virus*,[5] *Epstein—Barr virus*[6] and *Mycobacterium tuberculosis*[7] have been investigated by different authors with conflicting results.

It has been suggested that *Helicobacter pylori* (*H. pylori*) may be a risk factor of CAD.[8,9] *H. pylori* is a spiral-shaped, gram-negative bacteria that was discovered in 1983.[10] The association between CAD and *H. pylori* infection was reported for the first time by Mendall and colleagues in 1994.[11] This organism colonizes in the human stomach and causes gastritis, peptic ulcer and increases the risk of gastric carcinoma and gastric lymphoma. It is also found in the saliva, duodenum and feces.[12] Relationship between *H. pylori* and some extraalimentary tract disease is investigated by many researchers.[13—16] There is some evidence to support the idea that *H. pylori* may have a role in the development of atherosclerosis.[17—19] On the other side, some authors reported no association between *H. pylori* and atherosclerosis.[20—22] These different results emphasize the need for further research on the relationship between *H. pylori* and atherosclerosis.

Polymerase chain reaction (PCR) is a novel method for direct detection of microorganisms.[12] This study was performed to answer whether there are differences between coronary artery with and without atherosclerotic plaque in the presences of *H. pylori* DNA by polymerase chain reaction (PCR) method.

Methods

This case-control study was performed on 60 formalin fixed paraffin embedded (FFPE) of coronary artery biopsies in molecular pathology laboratory, Ghaem Hospital, Mashhad, Iran. Our samples included 30 coronary artery tissues with atherosclerosis plaques defined as a patient group, and 30 coronary arteries tissues without atherosclerotic plaques used as a control group. Coronary artery tissues were selected from samples of a previous study that was done in our university in determining prevalence of atherosclerotic plaques in autopsy cases with non-cardiac death in Northeast Iran.[23] Two pathologists investigated slides for confirming diagnosis and selecting the best paraffin blocks for DNA extraction. We reviewed medical history and previous laboratory reports of patients.

DNA extraction

Five to six 20-μm-thick sections were cut from each FFPE specimens under sterile conditions, and then the DNA was extracted using proteinase K extraction method.[24] DNA concentration was determined applying the Thermo Scientific NanoDrop 2000 Spectrophotometer and specimens with low DNA content (<20 ng/μL) were excluded from the study.

H. pylori detection

PCR was performed using the PCR Kits (DNA Technology JSC, PCR Kit, Moscow, Russia). 10 μl of PCR Master Mix and 0.5 μl unit of Taq polymerase (Hot Start Taq DNA Polymerase) were added into each paraffin-sealed tube and mixed. 5 μl (100 ng) of DNA samples were added (except for negative controls) and were spun at 1000 rpm for 3—5 s. The tubes were placed into Applied Biosystems (ABI) Veriti Thermal Cycler and polymerase chain reaction was started on a PCR Cycler with the 90 s at 94 °C for the first step and then 45 cycles was run as follows: 50 s at 94 °C, 50 s at 64 °C, 50 s at 72 °C and final extension were 10 min at 72 °C Positive control, which supplied by the manufacturer, were included in each reaction.

Amplified PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light by gel documentation machine. The results were interpreted as positive if we had DNA band corresponded to the band of the positive control [348-bp] in addition to internal control band [IC: 560 bp]. In other word, the result presumed as positive if there were two DNA bands, one of which corresponded to the DNA band of the positive control and the second corresponded to the DNA of the internal DNA band.

Statistical analysis

PCR results in normal and atherosclerotic groups were analyzed with SPSS (Statistical software for social analysis—version 11.5). We used Fisher's exact test for comparison of categorical variables and independent sample *t*-test for continuous variable in Case and control groups. A *P*-value below 0.05 ($P < 0.05$) was considered significant.

Results

The patients ranged in age between 20 and 46 years with an average age of 26 ± 6.41 years for mean ± standard deviation (SD). In patients group (case group), it was from 18 to 50 years with 32 ± 9.46 years for mean ± SD. Twenty-five patients (83.3%) were male and 5 patients (16.7%) were female. There is no differences in sex ratio (male to female ratio of 5:1) and no significant difference is seen between case and control groups for gender ($P = 1.00$) by Fisher's exact test. Comparisons of body mass index (BMI) in case and control groups were 24.4 ± 3.77 and 23.9 ± 2.62 kg/m^2, respectively [Table 1].

The frequency of atherosclerotic plaques in patients' heart vessels was shown in Table 2. Frequency of atherosclerotic plaques in arteries LCA(*left coronary artery*), RCA(*right coronary artery*) and LCX (*left circumflex artery*) groups of patients were 83%, 69% and 76%, respectively. As a result, the possibility of the existence of advanced plaque of atherosclerotic plaques in LCA was more frequent than two other vessels.

PCR results for detection of *H. pylori* DNA were negative in all samples of case and control groups. Fisher's exact Test showed no significant difference in detection of *H. pylori* DNA between samples of coronary artery with and without atherosclerotic plaques ($P = 1$).

Discussion

Arteriosclerosis is the main cause of obliterative vessel diseases such as cerebrovascular events and coronary artery disease. An acute vaso-occlusive event in heart and cerebral arteries is typically occurred by thrombosis formation at the site of arteriosclerotic plaque (endothelium) injury. It is well accepted that arteriosclerosis is primarily a chronic inflammatory process.[25] For the first time in 1988, Saikku et al. showed an association of an infectious agent (*C. pneumoniae*) with CAD in Finland.[26] Other infections may contribute to progression of this process.[25] It is postulated that serum antibodies to heat-shock protein correlated with *H. pylori* infection and suggested that immune reactions to HSP60 in atherogenesis are at least partially due to bacterial infections.[27]

Our results show that there is no correlation between *H. pylori* infection and development of coronary artery atherosclerosis. Similarly to our study, Sulewska et al.[28] and Kakkilaya et al.[29] studies indicated that there were not any *H. pylori* DNA in any atherosclerotic plaque by PCR method; and in other study, Latsios et al.[30] detected this organism only in 2.4% (very small portion) of their patients. Our findings are not concordant to the results of Ameriso et al.,[17] Giampaoloa et al.[18] and Kaplan et al.[19] studies that found significant association of previous infections by *H. pylori* with atherosclerosis. These discrepancies may be due to several causes including different methodology and epidemiology.

H. pylori can be detected by a variety of laboratory tests or techniques including organism culture, serum antibody measurement (serology), urease test (biochemical test), and histopathology (special stain).[10] Serology is one of the most available and least expensive methods for *H. pylori* detection.[31] Many of previous studies about *H. pylori* and atherosclerosis relation were based on this method. Nozari et al.,[32] Al-Ghamdi et al.[8] and Pieniazek et al.[33] found higher level of anti-*H. pylori* antibodies in CAD compared to control group but Ozdogru et al.,[34] Jha et al.[35] and Jia et al.[36] stated that the presence of *H. Pylori* antibodies in serum were unable to predict coronary artery events. We used PCR for detection of *H. pylori* infection which appears to be more specific than serology.[37] In Blasi et al.[38] and Hagiwara et al.[39] studies, more than 60% patients were seropositive for *H. pylori*; however they did not find any evidence of *H. pylori* DNA in atherosclerotic plaque by PCR method. Also similar to our findings, Ciervo et al.[40] did not find *H. Pylori* in any atherosclerotic plaque using real-time polymerase chain reaction (RT-PCR).

Epidemiology and geographic distribution are important factors that must be considered in making decisions about association of *H. pylori* and atherosclerosis. *H. Pylori* is a common pathogen worldwide especially in underdeveloped and developing countries. Infection with this organism is more common in adults and its prevalence varies between 7 and 87% and is lower in European studies.[41,42] Pinar and colleagues[43] detected *H. pylori* DNA in 20% of the normal group (non-atherosclerosis) by molecular method in Turkey that is neighbor of Iran. The *H. pylori* infection rate is common in the Iranian population.[44] Prevalence of this organism in normal population has been reported 58.6% in North of Iran by Bazzazi et al.[45] and 89% in Northwest Iran by Khadem Ansari et al.[46] They did not find any significant relationship between chronic infection of *H. pylori* and coronary heart disease by serologic method which is similar to our results in Northeast Iran.

Jha et al. reported more scores of percent positivity for *H. pylori* in aorta than carotid and coronary arteries by RT-PCR method.[2] Therefore, vascular location may be a minor affecting factor. In other study, Kilic et al.[47] demonstrated that there was a statistically significant difference between case and control groups for *H. pylori* DNA detection by PCR method in coronary and abdominal aorta arteries but in comparison to carotid arteries.

Table 1 Weight, height and BMI features in case and control groups.

	Patient group			Control group		
	Min	Max	Mean	Min	Max	Mean
Weight	44.00	90.00	67.40	45.00	95.00	72.56
Height	110.00	180.00	166	157.00	185.00	172.53
BMI	20.03	36.36	24.54	17.30	35.16	24.41

Table 2 The frequency of atherosclerotic plaques in heart vessels of patient group.

Atherosclerotic plaque	LCX	LCA	RCA
None	9(30%)	5(16.7%)	7(23.3%)
Fibro-fatty plaque	11(36.7%)	7(23.3%)	14(46.7%)
Advanced plaque	10(33.3%)	18(60%)	9(30.0%)

Conclusion

The results of our study showed that there is no significant relationship between coronary artery atherosclerosis and *H. pylori* infection using PCR method in Northeast Iran. More studies are needed with larger samples of cases (coronary artery atherosclerosis) and control groups to decrease sampling error.

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