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Detection of *Chlamydia pneumoniae* and *Helicobacter pylori* in atherosclerotic plaques of carotid artery by polymerase chain reaction

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Summary

Objectives: A possible role of some microorganisms has been proposed in the pathogenesis of atherosclerosis, but it is still an unresolved issue. We investigated the presence of *Chlamydia pneumoniae* and *Helicobacter pylori* DNA in carotid artery atherosclerotic plaques by using PCR.
Methods: One hundred and four patients with atherosclerotic diseases were included. The study group consisted of 52 atherosclerotic plaque specimens obtained from the carotid arteries of patients who had carotid endarterectomy and the control group consisted of 52 specimens obtained from the macroscopically healthy regions of ascending aorta in patients who had undergone coronary artery bypass grafting. The presence of *C. pneumoniae* and *H. pylori* DNA in endarterectomy specimens were demonstrated by PCR.
Results: *C. pneumoniae* DNA was detected in 16 of 52 (30.8%) atherosclerotic plaques and 1 of 52 (1.9%) macroscopically healthy ascending aorta wall specimens ($P < 0.001$). *H. pylori* DNA was detected in 9 of 52 (17.3%) atherosclerotic plaques and none of the controls ($P = 0.003$).

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Conclusions: The higher incidence of *C. pneumoniae* and *H. pylori* DNA in atherosclerotic plaques suggests that these microorganisms may play a role in the pathogenesis of atherogenesis.

Introduction

Atherosclerosis is defined as a chronic inflammatory disease. Risk factors in the formation, progression and destabilization of atherosclerotic plaques, including family history, hyperlipidemia, hypertension, diabetes mellitus, sex and smoking habits, cannot completely explain the pathogenesis of atherosclerosis, but only clarify some of its aspects.[1]

Recent data suggest a causative role for one or more infectious agents in some chronic inflammatory diseases such as duodenal ulcer, chronic respiratory diseases (including asthma) and atherosclerotic diseases.[2] The causal role of infectious agents in chronic inflammatory diseases and their association with these conditions have major implications related to public health, treatment and prevention. *Chlamydia pneumoniae* and *Helicobacter pylori* are among the most commonly implicated microorganisms in the pathogenesis of atherosclerosis.[3,4]

In this study, the presence of *C. pneumoniae* DNA and *H. pylori* DNA was investigated in carotid endarterectomy plaques and healthy ascending aorta wall specimens by polymerase chain reaction (PCR). Seroepidemiological findings are discussed in the light of the literature.

Methods

Study population

One hundred and four patients admitted to the Siyami Ersek Thoracic and Cardiovascular Surgery Center for surgery for atherosclerotic carotid or coronary artery disease between July 2000 and December 2001 were included in the study. Fifty-two atherosclerotic carotid plaque specimens from cases who had carotid endarterectomy constituted the study group (36 male, 16 female, mean age 67.5 $\pm$ 7.8) and 52 specimens from macroscopically healthy regions of the ascending aorta of cases who underwent coronary artery bypass grafting constituted the control group (38 male, 14 female, mean age 62.1 $\pm$ 8.6). Patients in the control group underwent operations for coronary artery disease, had no carotid atherosclerotic disease and the ascending aorta of these patients was macroscopically healthy. All patients in the study group were symptomatic and had carotid artery stenosis greater than 70%. Preoperative demographic characteristics and risk factors of cases were recorded. Risk factors for atherosclerosis including hypertension, diabetes mellitus, lipid profile, smoking habits, previous myocardial infarction and other medical history were recorded for each patient in both the study and the control groups.

Common risk factors for patients in the study and control groups are summarized in Table 1. Forty patients in the study group had only carotid endarterectomy, while 12 patients had both carotid endarterectomy and coronary artery bypass grafting operations. All 52 patients in the control group had aortocoronary bypass grafting operations.

Specimen collection

The specific recommendations of the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada) for PCR of *C. pneumoniae* were followed during the collection, transport, processing and assaying of specimens.[5]

All of the specimens were obtained in the operating room under sterile conditions. Carotid artery atherosclerotic plaque specimens of approximately 0.5—1 cm were placed into Eppendorf tubes containing Tris—EDTA buffer. Eppendorf tubes were labeled in the operating room. The microbiologists were blind with respect to the relevant groups (study and control) of specimens.

Specimen transport and processing

All of the specimens were sent to the laboratory and processed within 24 h; they were stored and transported at +4 °C. Calcified areas of vascular tissue specimens which are known PCR inhibitors were removed and the rest of the specimens were cut into small pieces ($\sim$25 mg) and processed for DNA extraction. All tissue samples were processed for PCR by proteinase potassium digestion, phenol—chloroform extraction, and ethanol precipitation. DNA extracts were formed into aliquots to avoid $>$1 freeze—thaw cycle for optimal yields.[5]

Table 1 The demographic data of the patients in the study and control groups.

Characteristics	Study group (n = 52)	Control group (n = 52)	P Value[a]
Mean age[b]	67.46 ± 7.84	62.08 ± 8.62	
Sex			0.665
Female	16 (30.77%)	14 (26.92%)	
Male	36 (69.23%)	38 (73.08%)	
Smoking habit	22 (42.31%)	21 (40.38%)	0.842
COPD	7 (13.46%)	3 (5.77%)	0.178
Coronary artery disease	25 (48.08%)	52 (100%); (group characteristic)	*<0.001*
Peripheral artery disease	5 (9.62%)	8 (15.38%)	0.372
Hypertension	39 (75%)	30 (57.69%)	0.061
Hypercholesterolemia	20 (38.46%)	18 (34.61%)	0.684
Total cholesterol level (mg/dL)	228 ± 48.12	239 ± 49.19	0.252
Obesity	5 (9.62%)	6 (11.54%)	0.750
Diabetes mellitus	10 (19.23%)	15 (28.85%)	0.250
Combination of risk factors			
Smoking + hypertension	16 (30.77%)	9 (17.31%)	0.106
Smoking + hypercholesterolemia	9 (17.31%)	2 (3.85%)	*0.021*
Smoking + hypertension + hypercholesterolemia	3 (5.77%)	1 (1.92%)	0.618

COPD, chronic obstructive pulmonary disease.
[a] $P < 0.05$ is significant.
[b] Mean ± standard deviation.

PCR amplification

DNA was amplified in 50 μl volumes containing 200 μM each deoxynucleoside triphosphate (dNTP) (MBI Fermentas, Burlington, Ontario, Canada), 2 μM of each primer, 2 U *Taq* polymerase (MBI Fermentas), 10 mM Tris—HCL, 50 mM KCl, 1.5 mM $MgCl_2$. Reaction tubes were placed in a thermal cycler (PTC-150 MJ Research, Waltham, MA, USA). Globulin primers were included in the assay as internal positive controls. Two positive and two negative controls were included in each assay as mix control.

The primer sequences, conditions, and size of these PCR methods are summarized in Table 2 for *C. pneumoniae*[5,6,7] and Table 3 for *H. pylori*.[8] For the detection of *C. pneumoniae* by PCR, two different PCR assays were done with two different sets of primers (Table 2).

Amplification products, 437—463 bp for *C. pneumoniae* and 294 bp for *H. pylori* were visualized by electrophoresis in a 2% agarose gel containing ethidium bromide. Tissue preparation, PCR amplification, and electrophoresis were performed in separate rooms, to avoid the risk of contamination.

Statistical analyses

Statistical analyses were carried out using SPSS 10.0 (SPSS Inc, Chicago, IL, USA) and MATLAB 6.0.88 Release 12 (The MathWorks, Inc, Boston, MA, USA). Data are expressed as mean ± standard deviation. A P value of less than 0.05 was considered to indicate statistical significance.

When statistically comparing the demographic data of the study and control groups, the Likelihood Ratio test and Fisher's Exact test based on the comparison of independent group ratios were used for sex, smoking habit, chronic obstructive pulmonary disease, coronary artery disease, peripheral artery disease, hypertension, hypercholesterolemia, obesity, diabetes mellitus, history of transient ischemic attack and unstable angina pectoris. For cholesterol

Table 2 PCR assays for *Chlamydia pneumoniae*.

PCR target gene, and size of PCR products	Primer names and sequences	PCR conditions and size of PCR products
(1) Cloned *PstI*, 437 bp	HL1 : 5′ gtt gtt cat gaa ggc cta ct 3′ HR1 : 5′ tgc ata acc tac ggt gtg tt 3′	94 °C-45 s, 48 °C-30 s, 72 °C-30 s (40 cycles)
(2) 16S rRNA, 463 bp	CpnA : 5′ tga caa ctg tag aaa tac agc 3′ CpnB : 5′ cgc ctc tct cct ata aat 3′	94 °C-1 min, 45 °C -30 s, 72 °C-30 s (40 cycles)

Table 3 PCR assay for *Helicobacter pylori*.

PCR Target gene, and size of PCR products	Primer names and sequences	PCR conditions
glmM gene, 294 bp	Forward, 5′ — aag ctt tta ggg gtg tta ggg gtt 3′	93 °C-1 min, 55 °C-1 min,
	Reverse, 5′ — aag ctt act ttc taa cac taa cgc 3′	72 °C-1 min (35 cycles)

levels, the Independent-Samples T test based on the comparison of independent two group means was used and homogeneity of cholesterol level variances were tested using Levene's f test. The Pearson Chi-square test and Odds Ratio (OR) test were used to investigate the probability of the presence of certain personal and environmental features in the study group patients with positive *C. pneumoniae* and *H. pylori*. Significance of OR values were tested by the calculation of 95% confidence interval (CI) limits and ranges not including '1' value were considered to be statistically significant ($P < 0.05$).

Results

The demographic data of the patients in the study group and the control group are shown in Table 1. No significant differences were found between the two groups with respect to age, sex and known risk factors (smoking habits, chronic obstructive pulmonary disease, peripheral artery disease, hypertension, hypercholesterolemia, obesity and diabetes mellitus).

Successful genomic DNA extraction was tested using globulin gene primers (data not shown). The extraction was performed a second time for three of the 104 samples to get DNA for successful amplification. The assay was validated using samples previously found positive for *H. pylori* or *C. pneumoniae*. The presence of *C. pneumoniae* was tested using two sets of primers and we observed complete concordance between methods. Two previously known positive and two negative controls were included in each assay as mix control against any PCR inhibitors or false positive amplification.

In the study group, the *C. pneumoniae* positivity rate was 30.77% (16 patients), whereas the *H. pylori* positivity rate was 17.31% (9 patients) (see Figures 1 and 2). However, in the control group, *C. pneumoniae* and *H. pylori* positivity rates were 1.9% and 0%, respectively. In one case, where right and left endarterectomies were performed separately 3 months apart, *H. pylori* was detected in both specimens. None of the samples were positive for both *C. pneumoniae* and *H. pylori*.

Results of the study and control groups regarding *C. pneumoniae* positivity were different and the difference was statistically significant (30.77% and 1.9% for study and control groups, respectively; $P < 0.001$ (2-sided), Pearson Chi-square test). The study and control groups also differed in terms of *H. pylori* positivity and the difference was also statistically significant ($P = 0.003$ (2-sided), Fisher's Exact test).

Demographic features of *C. pneumoniae* and *H. pylori* positive cases were investigated and the possibility of the presence of these features (Odds

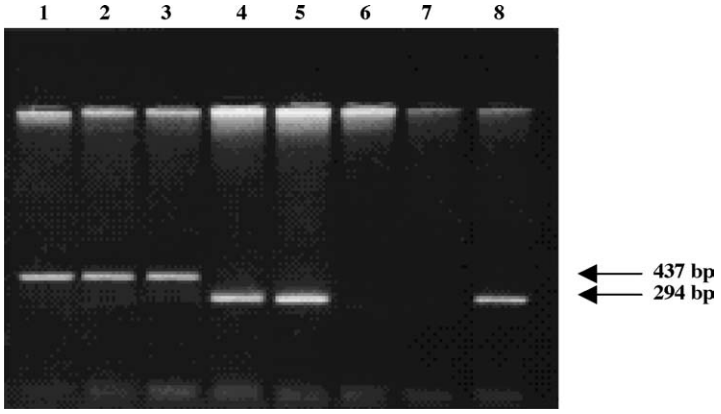

Figure 1 Amplification of cloned *PstI* gene of the *Chlamydia pneumoniae* and *glmM* (urease C gene) gene of the *Helicobacter pylori*. (+) control (*C. pneumoniae*): Lane 1; *C. pneumoniae* (+) patients: Lanes 2 and 3; (+) control (*H. pylori*): Lane 4; *H. pylori* (+) patients: Lanes 5 and 8; (−) control: Lanes 6 and 7.

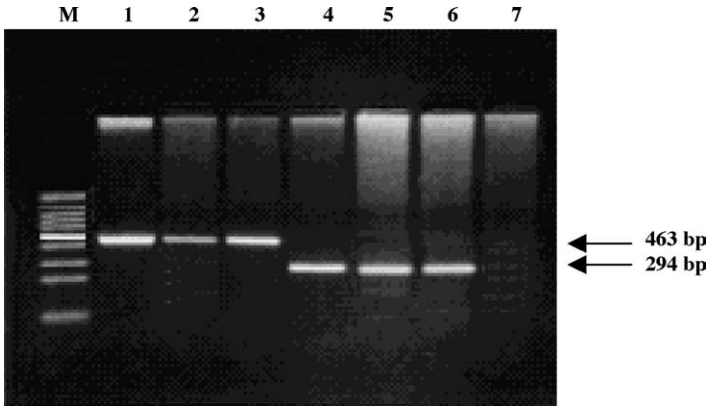

Figure 2 Amplification 16S rRNA gene of *Chlamydia pneumoniae* and *glmM* (urease C gene) gene of *Helicobacter pylori*. M: Marker: 100 bp ladder (Promega); (+) control (*C. pneumoniae*): Lane 1; *C. pneumoniae* (+) patients: Lanes 2 and 3; (+) control (*H. pylori*): Lane 4; *H. pylori* (+) patients: Lanes 5 and 6; (−) control: Lane 7.

Ratio) were calculated according to 95% CI values and they are shown in Table 4. Common features of study group patients positive for *C. pneumoniae* in their atheroma plaques are summarized in Table 4. In this group, in cases with *C. pneumoniae* positivity, the probability of the presence of coronary artery disease was increased 42.8 times, and probabilities were increased 13.6 and 3.55 times for the presence of peripheral artery disease and diabetes mellitus respectively, and these values were statistically significant ($P < 0.05$). Probability of chronic obstructive pulmonary disease was increased 1.53 times, hypertension and unstable angina pectoris 2.58 times and 3.37 times, respectively, however these were not statistically significant. Probabilities of other features were lower and the values were not statistically significant.

Common features of *H. pylori* positive patients are summarized in Table 4. The probability of chronic obstructive pulmonary disease was 2.02

Table 4 The demographic data of *Chlamydia pneumoniae* and *Helicobacter pylori* positive patients in the study group.

Characteristics	Study group ($n = 52$)		Odds Ratio (95% CI: lower < OR < upper)	
	CP positive	HP positive	CP	HP
Mean age[a]	66.4 ± 10.1	65.9 ± 8		
Sex				
Female	3	3	0.09 < 0.297 < 1.7[b]	0.25 < 0.90 < 5.33[b]
Male	13	6		
Atheroma plaque			$P = 0.630$[c]	$P = 0.603$[c]
Organized	8	7		
Soft	3	1		
Thrombosed	2	0		
Ulcerated	2	1		
Smoking habit	7	1	0.33 < 0.66 < 3.58[b]	0.01 < 0.14 < 1.14[b]
COPD	3	2	0.36 < 1.53 < 9.42[b]	0.35 < 2.02 < 13.5[b]
Coronary artery disease	15	1	4.5 < 42.8 < 335.3[d]	0.15 < 0.41 < 2.9[b]
Peripheral artery disease	4	1	1.18 < 13.6 < 114.9[d]	0.12 < 1.44 < 12.4[b]
Hypertension	14	5	0.56 < 2.58 < 15.9[b]	0.07 < 0.25 < 1.49[b]
Hypercholesterolemia	4	3	0.11 < 0.28 < 1.54[b]	0.17 < 0.59 < 3.4[b]
Obesity	1	1	0.05 < 0.62 < 5.19[b]	0.12 < 1.44 < 12.4[b]
Diabetes mellitus	6	1	1.13 < 3.55 < 20.5[d]	0.05 < 0.53 < 4.28[b]
TIA history	8	4	0.48 < 0.95 < 5.15[b]	0.26 < 0.82 < 4.72[b]
Unstable angina pectoris	2	1	0.14 < 3.37 < 39.8[b]	0.29 < 7.69 < 92.8[b]

COPD, chronic obstructive pulmonary disease; CP, *C. pneumoniae*; HP, *H. pylori*; OR, Odds Ratio; TIA, transient ischemic attack.
[a] Mean ± standard deviation.
[b] 95% CI, $P > 0.05$.
[c] $P < 0.05$ is significant, (2-sided), Pearson Chi-square test.
[d] 95% CI, $P < 0.05$.

times higher, the probability of peripheral arterial disease was 1.44 times higher, the probability of obesity was 1.44 times higher and the probability of unstable angina pectoris was 7.69 times higher, however these were not statistically significant. Probabilities of other features were lower but values were not statistically significant.

Discussion

An association between infectious processes and atherosclerosis has been reported in numerous studies in recent years.[1] *C. pneumoniae* and *H. pylori* are among the most frequently implicated microorganisms.[3]

By using the PCR method, we investigated the presence in atherosclerotic plaques of *C. pneumoniae*, which is the most strongly implicated, and *H. pylori*, a common infectious agent in our country. A possible association between *C. pneumoniae* and atherosclerosis was first demonstrated by a seroepidemiological study by Saikku and colleagues in 1988.[9] Shore and colleagues[10] first demonstrated the presence of *C. pneumoniae* in atheroma plaques by using the PCR method and electron microscopy. Although *C. pneumoniae* positivity in atheroma plaques was demonstrated in most of the studies which used the PCR method following the study of Shore and colleagues, rates varied considerably.[3,10–13] The most significant cause for this is lack of standardization with regard to PCR methods. In a study by Apfalter and colleagues,[14] the presence of *C. pneumoniae* was investigated in 15 endarterectomy specimens and each specimen was investigated at nine different centers by PCR. *C. pneumoniae* positivity for each specimen varied between 0 and 60% (intercenter variability). Reasons for obtaining such variable results by PCR are suggested to include patchy distribution of *C. pneumoniae* in atherosclerotic lesions, insufficient atheromatous tissue examined, lack of standardization of laboratory methods, inability to perform *C. pneumoniae* DNA extraction from atheroma plaques and the presence of PCR inhibitors in atheroma plaques.[15,16]

In order to provide a standard for *C. pneumoniae* PCR tests and to exclude possible factors that may prevent the detection of *C. pneumoniae* by PCR, the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada) published a guideline named ''Standardizing *Chlamydia pneumonaie* assays''.[5] In our study, we performed *C. pneumonaie* PCR according to the recommendations of this guideline in obtaining the specimens, transportation, extraction and for PCR processes and we used two of the four methods[6,7] that were designated as standard in this guideline. The *C. pneumoniae* positivity rate was found to be 30.77% by both of these methods. The *C. pneumoniae* positivity rate in atheroma plaques was reported as 26% in a Turkish study, in which 16S rRNA primers were also used.[17] In a study from Washington University, which used the other primer of our study, the *C. pneumoniae* positivity rate in carotid artery plaques was found to be 24%[18] by PCR. In a report that evaluated the results of 30 other studies that investigated the presence of *C. pneumoniae* in atheroma plaques obtained from arterial samples by PCR, the overall *C. pneumoniae* positivity rate was 25% for atheroma plaques, whereas it was 1% or less for normal vascular tissues.[15] The *C. pneumoniae* positivity rate of 30.77% in carotid atheroma plaques that we found in our study also supports these results.

In a number of studies, some clinical features of atherosclerosis[12,19] were found to be associated with *C. pneumoniae* positivity in atherosclerotic tissues. In our study there were no differences between *C. pneumoniae* positive and negative cases with respect to these kinds of lesions. But coronary artery disease, peripheral arterial disease and diabetes mellitus were found to be more frequent in *C. pneumoniae* positive cases than negative cases, and these were statistically significant ($P < 0.05$). Establishing a possible causal relationship between *C. pneumoniae* and these diseases from our results is difficult and further studies concerning this subject are required.

Another microorganism that is suggested to play a role in the pathogenesis of atherosclerosis is *H. pylori*. *H. pylori* is a Gram-negative, spiral bacillus; its causal role in duodenal ulcer has been demonstrated.[2] There is not yet evidence as strong for an association between *H. pylori* and atherosclerosis as there is for *C. pneumoniae*. However in countries with an *H. pylori* prevalence of greater than 80%,[20] such as Turkey, it is important to clarify this issue. Most studies demonstrating an association between *H. pylori* and atherosclerosis are serological studies. Such an association was reported for the first time by Mendall and colleagues.[21] The high prevalence of *H. pylori* infections in developing countries (e.g. Turkey) devalues serological studies. Because *H. pylori* seropositivity will be above 80% in any control group, comparison with study groups will be impossible. Therefore, in our study we directly investigated the presence of *H. pylori* in atheroma plaques by PCR. The results of the studies investigating the presence of *H. pylori* in atherosclerotic plaques by PCR are conflicting.[22–29] In some of these studies, the presence

of *H. pylori* could not be detected,[22–26] whereas in others the presence of *H. pylori* could be shown.[17,27–29] Standardization problems related to PCR may have a role in the failure to detect *H. pylori* in atheroma plaques by the PCR method, as is the case for *C. pneumoniae*.

The *glmM* (urease C) primer that we used in our study for the detection of *H. pylori* is currently regarded as the most specific primer.[30] In studies where *H. pylori* was investigated in atheroma plaques by the PCR method, positivity rates varied between 22 and 51%.[17,27–29] In our study, *H. pylori* positivity in atheroma plaques was 17.31% compared to 0% in healthy vascular tissues, and the difference was statistically significant ($P = 0.003$). In addition, a patient had right and left carotid endarterectomy operations 3 months apart and *H. pylori* was positive in both atheroma plaques obtained from right and left sides, suggesting that *H. pylori* causes a systemic effect on atherogenesis.

Socioeconomic level is known to be associated with *H. pylori* infections.[31] Two of five studies which demonstrated the presence of *H. pylori* (including our study) are reported from Turkey[17] and one other from Argentina[27] which is also a developing country, suggesting that this organism may play a role in atherosclerosis where the prevalence of this infection is high.

In our study *C. pneumoniae* and *H. pylori* were found at a greater rate in carotid artery atheroma plaques, compared to healthy vascular walls (for *C. pneumoniae* $P < 0.001$; for *H. pylori* $P = 0.003$). These results support the suggestion that both *C. pneumoniae* and *H. pylori* have a role in the pathogenesis of atherosclerosis.

Conflict of interest: No conflict of interest to declare.

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