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Detection of *Chlamydia pneumoniae* and *Helicobacter pylori* DNA in Human Atherosclerotic Plaques by PCR

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***Chlamydia pneumoniae* and *Helicobacter pylori* can cause persistent infections of the respiratory and gastrointestinal tract, respectively. It has been suggested that persistent infection of arteries with these bacteria can contribute to the development of atherosclerosis. The aims of this study were to determine the presence of *C. pneumoniae* and *H. pylori* DNA in atherosclerotic plaque samples by PCR and to evaluate the correlation between clinical status and DNA positivity of these bacteria. Eighty-five consecutive patients (mean age, 59 ± 10; 75 male, 10 female) undergoing coronary artery bypass grafting, carotid endarterectomy, and surgery of the abdominal aorta for atherosclerotic obstructive lesions were included in the study. Forty-six endarterectomy specimens from the atherosclerotic lesions and 39 specimens from healthy regions of the ascending aorta, which were accepted as the control group, were excised. The presence of microorganism DNA in endarterectomy specimens was assessed by PCR. *C. pneumoniae* DNA was found in 12 (26%) of 46 endarterectomy specimens and none of the healthy vascular-wall specimens ($P < 0.001$), while *H. pylori* DNA was found in 17 (37%) of 46 endarterectomy specimens and none of the controls ($P < 0.001$). Either *C. pneumoniae* or *H. pylori* DNA was positive in 23 (50%) of 46 patients and none of the controls ($P < 0.001$). Six of the atherosclerotic lesions showed coexistence of both of the microorganism DNAs. The presence of *C. pneumoniae* and *H. pylori* DNA in a considerable number of atherosclerotic plaques but their absence in healthy vascular wall supports the idea that they may have a role in the development of atherosclerosis, especially in countries where infection is prevalent and where conventional risk factors fail to explain the high prevalence of atherosclerotic vascular disease.**

Conventional risk factors, including hyperlipidemia, hypertension, diabetes, tobacco use, sex, and family history of premature vascular disease, account only for approximately half of the patients with clinically apparent atherosclerosis (22). Recently, a potential link between infectious agents and atherosclerosis has been suggested. Data obtained from several seroepidemological studies has given rise to the hypothesis that an infection can initiate or maintain the atherosclerotic process (8). Pathophysiological mechanisms by which this may occur have been described in experimental studies (2, 16) and include effects on lipid metabolism, leukocyte–endothelial-cell interaction, coagulation factors, and platelet activation. Infections caused by *Chlamydia pneumoniae* and *Helicobacter pylori* have been postulated to be of interest (15). Seroepidemiological evidence, immunocytochemisty, and molecular biology studies have suggested an association between *C. pneumoniae* and coronary artery disease. However, epidemiological and serological data suggesting an association between *H. pylori* and atherosclerosis are conflicting. Furthermore, seropositivity does not correlate with the presence and extent of atherosclerosis.

In the present study, the presence of *C. pneumoniae* and *H. pylori* DNA were investigated by PCR in endarterectomy and vascular-wall specimens, as was the correlation between the clinical status and DNA positivity of these bacteria.

MATERIALS AND METHODS

Patients. Eighty-five consecutive patients admitted to the Department of Cardiovascular Surgery, patients with various manifestations of ischemic vascular disease and all undergoing surgery, were included in the study. The inclusion period was from January to November 1998. Demographic characteristics, smoking habits, lipid profile, and medical history were recorded for each patient. The patients were further evaluated to assess whether they were clinically stable or unstable. Patients who had a recent acute coronary syndrome or transient ischemic attack were characterized as unstable. The remaining patients were accepted as clinically stable. Forty-six specimens (lesion group) from atherosclerotic lesions (10 coronaries, 18 carotids, and 18 abdominal aortas) and 39 specimens (control group) from the macroscopically healthy regions of the ascending aorta were obtained.

Specimen collection. All specimens were dissected in the operating room under sterile conditions. Artery segments approximately 4 to 5 mm in length were placed in microcentrifuge tubes containing Tris-EDTA buffer. Transport vials were sealed in the operating room and opened only in the laminar air flow safety cabinet at the microbiology laboratory. All of the specimens were kept at −20°C until processing. Dissected arterial materials were ground by a sterile glass grinder. Chromosomal DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method according to the DNA Miniprep protocol of Wilson (30). This method is known to remove complex polysaccharides which may inhibit PCR amplification.

PCR amplification. (i) *C. pneumoniae*. For the detection of *C. pneumoniae* by PCR, primers that amplify 463-bp fragment of the 16S rRNA gene were used (10). After amplification, 1.2% agarose gel electrophoresis at 100 V and ethium bromide staining were used to visualize the PCR products.

(ii) *H. pylori*. The primers HPU1 and HPU2 were used to amplify a 411-bp internal fragment of the urease A gene of *H. pylori* (7). This assay has been assessed previously for its specificity for the urease A gene of *H. pylori* and found not to cross-react with other *Helicobacter* species or other known urease-producing organisms (1). Agarose gel (1.2%) electrophoresis at 100 V and ethidium bromide staining were used to visualize the PCR products.

All of the *H. pylori*-positive samples were tested by nested PCR for the 16S rRNA gene of *H. pylori* for confirmation. The primers HP1 and HP3 that amplify 446-bp fragment of 16S rRNA gene were used. After the initial reaction, HP1 and HP2 primers were used for the amplification of the nested 109-bp fragment (18). Additionally, all of the positive samples for *C. pneumoniae* and *H. pylori* and some randomly selected negative vascular samples were tested by PCR with primers that amplify the 123-bp fragment of the repetitive sequence of *Mycobacterium tuberculosis* as controls (14).

PCR was performed at least two times on different days for each bacterium. The microbiologists were blinded to the pathology and clinical status of the patients.

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TABLE 1. Characteristics of patients in the lesion group and the control group

Parameter	Lesion group (n = 46)	Control group (*n* = 39)	*P*
Mean age ± SD	60 ± 9	57 ± 10	0.150
Sex (no. male/no. female)	42/4	33/6	0.343
No. of patients (%) with:			
Hypertension	20 (43)	17 (37)	0.992
Diabetes	8 (17)	12 (26)	0.150
Smoking history	34 (74)	22 (49)	0.092
Family history	7 (15)	12 (26)	0.088
Total cholesterol level (mean mg/dl ± SD)	228 ± 70	204 ± 44	0.064

Statistical analysis. Statistical analysis was performed with SPSS 7.5 for Windows. Continuous variables were analyzed with the two-sample *t* test (in the case of normal distribution) or the Mann-Whitney U rank-sum test. Binary data were analyzed with Fisher's exact test. All tests were two tailed. A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

The demographic data of the patients in the lesion group and the control group are listed in Table 1. No significant differences were found between the two groups with respect to age, sex, known risk factors, and cholesterol levels.

C. pneumoniae DNA was positive in 26% (12 of 46) of the atherosclerotic lesions but in none of the healthy vascular wall specimens ($P < 0.001$), while *H. pylori* DNA was found in 37% 17 of 46) of the lesions and in none of the controls ($P < 0.001$). Either *C. pneumoniae* or *H. pylori* DNA was positive in 50% (23 of 46) of the lesions and in none of the healthy vascular wall specimens ($P < 0.001$). Six of the atherosclerotic lesions, four from the abdominal aorta and two in a coronary location, showed both *C. pneumoniae* and *H. pylori* DNA positivity. As shown in Tables 2 and 3, the demographic characteristics of the patients did not differ with respect to *C. pneumoniae* and *H. pylori* DNA positivities.

All of the samples tested for confirmation by 16S rRNA *H. pylori* PCR were found to be positive. The *M. tuberculosis* PCR was negative for all samples that tested.

Totals of 11 of the 18 specimens obtained from the abdominal aorta, 7 of the 18 specimens obtained from the carotids, and 5 of the 10 specimens from coronaries were found to be positive for at least one of the organisms. Neither *C. pneumoniae* nor *H. pylori* showed any location preference (for *C. pneumoniae,* $P = 0.279$; for *H. pylori,* $P = 0.242$) (Table 4).

TABLE 2. Clinical characteristics of patients with respect to *H. pylori* DNA positivity

Parameter	Patients that are *H. pylori*:		*P*
	Positive (*n* = 17)	Negative (*n* = 29)	
Mean age ± SD	61 ± 10	60 ± 9	0.803
Sex (no. male/no. female)	15/2	27/2	0.576
No. of patients (%) with:			
Hypertension	4 (24)	16 (55)	0.039
Diabetes	3 (18)	5 (17)	0.972
Smoking history	10 (59)	24 (83)	0.078
Family history	3 (18)	4 (14)	0.728
Total cholesterol level (mean mg/dl ± SD)	219 ± 63	241 ± 70	0.306

TABLE 3. Clinical characteristics of patients with respect to *C. pneumoniae* DNA positivity

Parameter	Patients that are *C. pneumoniae*:		*P*
	Positive (*n* = 12)	Negative (*n* = 34)	
Mean age ± SD	62 ± 10	60 ± 9	0.501
Sex (no. male/no. female)	12/0	30/4	0.219
No. of patients (%) with:			
Hypertension	5 (42)	15 (44)	0.884
Diabetes	1 (8)	7 (20)	0.341
Smoking history	10 (83)	24 (70)	0.393
Family history	2 (17)	5 (15)	0.872
Total cholesterol level (mean mg/dl ± SD)	201 ± 78	237 ± 64	0.119

Twenty-eight patients undergoing coronary artery bypass grafting or carotid endarterectomy were evaluated further for the presence of unstable plaque. Totals of 3 of 10 patients in the coronary artery disease group and 14 of 18 patients in the carotid disease group had unstable clinical characteristics. One of these three clinically unstable patients in the coronary group showed positivity for *H. pylori*, and one other showed positivity for both microorganisms. In the carotid group, 6 of 14 clinically unstable patients show were positive for either one of these microorganisms (four for *H. pylori* and two for *C. pneumoniae*). A correlation between *C. pneumoniae* and/or *H. pylori* positivity and the stability of the atherosclerotic lesions was not found ($P = 0.435$). No relation was found between total cholesterol level and *C. pneumoniae* and/or *H. pylori* positivity.

DISCUSSION

C. pneumoniae, an obligate intracellular gram-negative bacterium, has been associated with atherosclerotic cardiovascular disease both by seroepidemiological studies, indicating a significantly higher prevalence of circulating *C. pneumoniae* antibody or immune complexes among persons with clinical or radiographic evidence of atherosclerotic disease (26, 28). The organism has been detected by electron microscopy, immunocytochemisty, direct immunofluorescence, and the PCR in coronary artery (5, 21), aorta (3), and carotid artery (11, 13) plaque specimens, and it has been cultured from the coronary artery of a patient with coronary atherosclerosis (25). Further evidence supporting a causative role for *C. pneumoniae* in clinical outcomes related to coronary artery disease comes from the ROXIS study, which reported the favorable effects of the antichlamydial antibiotic roxithromycin in patients with angina and non-Q-wave myocardial infarction (12). In our study, the presence of *C. pneumoniae* in 26% of the atherosclerotic lesions but none of the healthy vascular wall supported their possible role in atherogenesis. However, their

TABLE 4. Localization of *H. pylori* and *C. pneumoniae* in the vascular tree

Site (*n*)	No. of isolates found		
	C. pneumoniae	*H. pylori*	Both
Coronary (10)	2	5	2
Abdominal aorta (18)	7	8	4
Carotid (18)	3	4	0
Healthy segment (39)	0	0	0

absence in nearly three-fourths of the lesions shows that they are not an obligate component of the atherosclerotic process. They might have effects on the initiation and/or acceleration of an ongoing process. In our study, we found no association between clinical instability defined as acute coronary syndrome or transient ischemic attack and the presence of *C. pneumoniae*.

H. pylori is an agent of chronic infection of the human stomach, and almost half of the adult population has serological evidence of infection with *H. pylori*. Recently, *H. pylori* seropositivity has been shown to be associated with coronary artery disease (19). However, early findings of an increased prevalence of *H. pylori* seropositivity among men with angiographically confirmed ischemic heart disease was criticized due to selection bias and unmeasured socioeconomic factors. Proposed mechanisms for how *H. pylori* might increase risk of coronary heart disease include increased plasma fibrinogen, C-reactive protein, blood leukocyte count, and homocysteine in seropositive subjects (24–27). In 1996, genomic material of *H. pylori* was demonstrated by PCR in the coronary arteries of a few subjects with myocardial infarction at autopsy (A. Cunningham, M. Ward, R. Matthews, and R. Ellis, Abstr. 1st Eur. Cong. Chemother., abstr. W149, 1996). Recently, Danesh et al. have shown *H. pylori* genome in buffy coat samples and diseased arterial segments; theirs is the first study to demonstrate the presence of *H. pylori* genomic material in living subjects (9). Meanwhile, two studies failed to demostrate any evidence of *H. pylori* in the atherosclerotic plaques of abdominal aortic aneurysms (4) and carotid arteries (6) of patients who were seropositive for *H. pylori*. Furthermore, the presence of serum antibodies does not necessarily indicate the persistence of active infection at any site or persistent exposure of the vascular system to any type of insult. Our study is the second to demonstrate the presence of *H. pylori* in atherosclerotic plaques in living subjects.

Although the urea A primers used for the detection of *H. pylori* are highly specific and sensitive, we confirmed our findings by using 16S rRNA nested PCR. Additionally, we checked the *H. pylori-C. pneumoniae*-positive samples and randomly selected negative samples for *M. tuberculosis* DNA in order to be sure of our positive results. *M. tuberculosis* is an intracellular microorganism which is prevalent in our country. The negative results showed that the PCR assays we used are reliable.

The failure of the previous studies to show the presence of *H. pylori* in atherosclerotic segments despite the presence of antibodies may be due to the different ethnicities of the study groups. *H. pylori* seropositivity in our country is high. In a recent study the seropositivity of blood donors in our hospital was found to be 84% (Y. Akyön, M. Bennedsen, O. İ. Özcebe, E. Bayerdorffer, and L. P. Andersen, Abstr. 10th Int. Workshop CHRO, abstr. HE14, 1999). *H. pylori* infection is thought to be more prevalent in underdeveloped regions, among patients with lower folate and higher homocysteine levels. Previous studies in our country have indicated that the population has low high-density lipoprotein, high homocysteine, and low folate levels (17, 29).

Among different segments of the vascular tree, we found no site preference for the microorganisms. DNA positivity for both bacteria was found in six of the specimens. This cannot be explained by contamination since meticulous care was taken to perform all processing under DNA-free cabinets with laminar air flow and all specimens were amplified twice. The coexistence of both microorganisms did not affect the presence of acute events in these patients.

The presence of these microorganisms in a considerable number of atherosclerotic plaques but their absence in all of normal vasculature and the negative results of *M. tuberculosis* PCR suggest a new evidence for the role of these microorganisms in atherogenesis rather than being just an 'innocent bystander.' The present study suggests that *C. pneumoniae* and *H. pylori* have an important role in atherosclerosis pathogenesis, especially in Turkey, where infection is prevalent and conventional risk factors fail to explain the high prevalence of atherosclerotic vascular disease.

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