

# Detection of *Helicobacter pylori* and *Chlamydia pneumoniae* DNA in human coronary arteries and evaluation of the results with serologic evidence of inflammation

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## ABSTRACT

**Objectives:** Atherosclerosis is pathologically similar to a chronic inflammatory response. Recent reports have suggested that *Chlamydia pneumoniae* (*C. pneumoniae*) and *Helicobacter pylori* (*H. pylori*) play a role in the pathogenesis of atherosclerosis but this relation has not been confirmed on an inflammatory background.

**Methods:** Twenty-nine consecutive patients admitted to Suleyman Demirel University Medical School Cardiovascular Surgery Department, Isparta, Turkey between May 2002 to June 2003 were included in the study and the presence of *C. pneumoniae* and *H. pylori* DNA in atherosclerotic plaques of 14 coronary endarterectomy specimens and 15 left internal mammary artery (LIMA) specimens as control subjects were determined by polymerase chain reaction. Serologic evidence of infection and inflammatory markers were also determined in both groups.

**Results:** Two *C. pneumoniae* DNA cases from the plaque group (14.3%) and 4 *H. pylori* DNA cases; 3 from plaque (21.4%) and one from the LIMA groups (6.7%)

were detected. The C-reactive protein (mg/L) were higher in DNA positive samples of *C. pneumoniae* (66.58) and *H. pylori* (21.93) compared to DNA negatives of *C. pneumoniae* (8.49) and *H. pylori* (10.98), similarly interleukin-6 (U/L) levels were higher in DNA positive samples of *C. pneumoniae* (42.25) and *H. pylori* (56.37) compared with DNA negatives of *C. pneumoniae* (17.52) and *H. pylori* (13.28), but the differences were not statistically significant. Apolipoprotein B levels were significantly higher in *C. pneumoniae* immunoglobulin M positive cases (0.844 g/L) compared with negatives (0.661 g/L) ( $p=0.004$ ).

**Conclusion:** Chronic infections modify the serum lipid profile in a way that increases the risk of atherosclerosis. The increased titers of inflammation markers in DNA positive patients support inflammation in atherosclerosis, however, the results should be reproduced in a larger cohort.

Saudi Med J 2005; Vol. 26 (7): 1068-1074

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**R**elations between atherosclerosis, C-reactive protein (CRP)<sup>1</sup> and interleukin 6 (IL-6)<sup>2</sup> indicate chronic inflammation, which may be caused by a low grade infection by certain bacteria. Interleukin 6, which is the major cytokine of the acute phase response is predictive of an initial cardiovascular

event,<sup>3</sup> and the primary determinant of CRP production.<sup>4</sup> Increased high sensitive CRP (hs-CRP) levels have been associated with increased mortality from coronary artery disease (CAD) in patients with stable angina.<sup>3</sup> Results of many studies show that apolipoprotein B (apo B) is a better marker of risk

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Received 15th January 2005. Accepted for publication in final form 12th April 2005.

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of vascular disease than other lipid markers.<sup>5</sup> Our aim was to determine the direct association between atherosclerosis and *Chlamydia pneumoniae* (*C. pneumoniae*) and *Helicobacter pylori* (*H. pylori*) infections by detecting specific DNA in atherosclerotic plaques, and left internal mammary arteries (LIMA) as a control group. We also searched for a proof of inflammation by investigating the correlation between DNA positivity in plaques, and CRP, IL-6; and apo B levels in serum.

**Methods.** The study was designed as a case control study. Twenty-nine consecutive patients admitted to the Suleyman Demirel University Medical School Cardiovascular Surgery Department between May 2002 to June 2003, undergoing surgery with various manifestations of ischemic heart diseases were included in the study. Fourteen coronary endarterectomy specimens as the atherosclerotic group, and 15 LIMA specimens without any plaques as controls were collected. All experiments were performed in compliance with the relevant laws and institutional guidelines. After the details of the research had been explained, the participants' informed consent was obtained before the operation. Coronary endarterectomy was performed in patients who would not have good distal perfusion without endarterectomy, or would not achieve graft patency and adequate anastomosis quality. Coronary endarterectomy patients' vessels had severe atherosclerotic involvement. The LIMA was chosen as the control group artery as this artery very seldom contains atherosclerotic plaques and arteries with plaques were not included in this study. The LIMA specimens were collected from patients undergoing coronary bypass operation. The patients' age, gender, cigarette smoking, patients diagnosed as diabetes mellitus (DM), hypertension (HT), hypercholesterolemia (HCL) and obesity were recorded. Coronary artery disease risk factor determinations and definitions were derived from the study of Ossei-Gerning et al.<sup>6</sup> All specimens were dissected in the operating room under sterile conditions. Coronary artery segments and LIMA specimens of one cm in length were placed into sterile tubes containing sucrose phosphate glucose solution (7.5% sucrose, 0.052% KH<sub>2</sub>PO<sub>4</sub>, 0.1529% Na<sub>2</sub>HPO<sub>4</sub> 2H<sub>2</sub>O and 0.072% glutamic acid). Tubes were sealed in the operating room and opened only in the laminar airflow cabinet at the microbiology laboratory. Blood samples from each patient were collected and sera were obtained. All of the specimens were kept at -80°C until analysis. The calcified and fatty tissue parts of the arteries were discarded and the remaining segments of the arterial materials were chopped by lancet in sterile conditions and were incubated in 0.1 mg/ml

proteinase lysis solution (150 mM NaCl, 25 mM EDTA, 10 mM Tris-HCl pH 8.0, 0.5% SDS) at 65°C for 3 hours. After purification of DNA by phenol-chloroform extraction, DNA was precipitated in 50 mM Na ethanol at -20°C overnight. Phenol-chloroform was chosen to get rid of the lipid layer of atheromatous plaques. After drying, DNA precipitates were suspended in 50 µl sterile deionated water of which 5 µl were used for amplification. The extraction method was modified from Blasi et al.<sup>7</sup>

Amplification by polymerase chain reaction (PCR) was successfully performed using 2 sets of primers that amplify 207 bp region of *ompA* gene locus of *C. pneumoniae* by nested PCR.<sup>8</sup> External primer set which amplified 333 bp was CP1:(sense) 5' TTA CAA GCC TTG CCT GTA GG 3', CP2 (anti-sense): 5' GCG ATC CCA AAT GTT TAA GGC 3' and the internal primer set which amplified 207 bp was CPC (sense): 5' TTA TTA ATT GAT GGT ACA ATA 3' and CPD (anti-sense): 5' ATC TAC GGC AGT AGT ATA GTT 3'.<sup>8</sup> The *C. pneumoniae* strain (from Metis Biotechnologies Inc.) was used as a positive control for the whole procedure. Reaction mixture without DNA was used as a negative control. Five µl of the extracted sample DNA was subjected to PCR under mineral oil overlay in the same PCR buffer with 0.4 µM each of CP1 and Cp2 primers, 50 mM KCl, 10 mM Tris-HCl (pH:8.3) 2.5 mM MgCl<sub>2</sub> solution, containing 200 µM dNTP's of dATP, dCTP, dGTP and dTTP each and one unit Taq DNA polymerase gold enzyme. After amplification of the PCR products by the outer primers, they were diluted 10 times in distilled water, and 10 µl was transferred to a PCR reaction mix for amplification in a second stage using the inner primers. The PCR reaction mix was the same as before except from each of the primers one µM CPC and CPD was used. This method was modified from Tong et al.<sup>8</sup> For *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).<sup>9</sup> The *H. pylori* ATCC 43629 strain was used as a positive control for the whole procedure. Reaction mixture without DNA was used as a negative control. The first amplification was performed with the Hp1 and Hp3 primers of 3 µl each in 50 µl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH:8.3) 2.5 mM MgCl<sub>2</sub> solution, 200 µM dNTP's of dATP, dCTP, dGTP and dTTP each, and 1 unit Taq DNA polymerase gold enzyme. The reaction mixture was overlaid

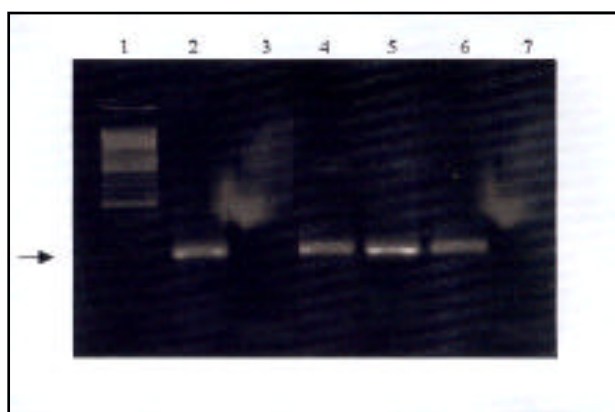


Figure 1 - Amplification products of *Chlamydia pneumoniae* positive, negative and control samples in agarose gel. Lane 1, 100 bp molecular weight marker; lanes 2 and 4, positive samples; lane 3, negative sample; lanes 5 and 6, positive control and lane 7 negative control. Arrow shows 207 bp marker.

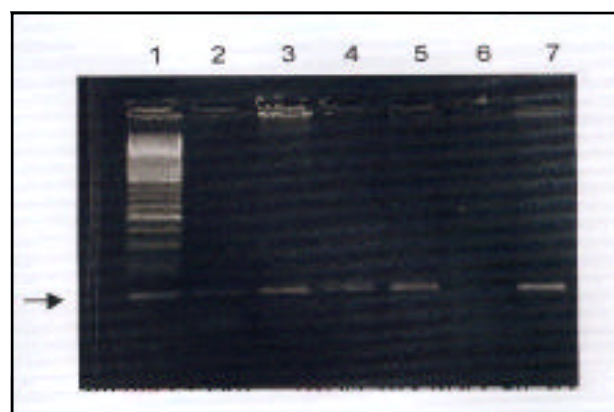


Figure 2 - Amplification products of *Helicobacter pylori* positive, negative and control samples in agarose gel. Lane 1, 100 bp molecular weight marker; lanes 2,3,4 and 5, positive samples; lane 6, negative sample; and lane 7, positive control. Arrow shows 109 bp markers.

with mineral oil. After amplification as described below, one  $\mu$ l of the primary amplification product was used in a 50  $\mu$ l reaction mixture with primers Hp1 and Hp2 under the same conditions described above. This method was modified from Dowsett et al.<sup>9</sup> Amplification was performed in MJR PTC 100 "thermalcycler" (MJR, USA).

Amplifications of *C. pneumoniae* and *H. pylori* were performed under identical conditions. After pretreatment of the samples at 95°C for 5 minutes, 30 cycles of amplification were performed. Each cycle consisted of annealing at 55°C for 45 seconds, elongation at 68°C for one minute and denaturation at 95°C for 30 seconds. Agarose gel (3%) electrophoresis at 100 V and ethidium bromide staining were used to visualize the PCR products by standard techniques.<sup>10</sup> A ultraviolet transilluminator (Kodak EDAS 290, USA) was used to compare the amplified samples with 100 bp mw marker band (Figures 1 & 2). All samples were tested blindly and repeated at least twice before reporting. All sera samples were tested for *H. pylori* immunoglobulin (Ig)G and IgA and *C. pneumoniae* IgG and IgM and evaluated qualitatively by the enzyme linked immunoassay (ELISA) method (Euroimmun GmbH, Am-Sonneberg, Germany). Levels above 20 'relative units/ml' were accepted positive for qualitative assessment according to the manufacturer's instructions. Serum IL-6 (Cytoscreen Diagnostics, USA) concentrations were detected quantitatively by ELISA method. Apolipoprotein B (Dade Behring, Schwalbach, Germany) and CRP levels (N High-sensitivity CRP, Dade Behring; Schwalbach, Germany) were detected using the particle enhanced immunonephelometry method. C-reactive protein levels were analyzed by high sensitive CRP method,

which detects serum CRP levels as low as 0.1 mg/l. Categorical data were analyzed by chi-square test or the Fischer's exact test for small samples. Differences in means of continuous variables between groups were compared by Student's t-test.  $p < 0.05$  were accepted as significant. Statistical analyses were performed using the Statistical Package for Social Sciences version 10 for Windows software packet (SPSS Inc., Chicago, Ill, USA).

**Results.** The demographic data of the patients in the atherosclerotic and control groups are listed in **Table 1**. No significant differences were found between the groups with respect to age, gender and known risk factors of atherosclerosis. The *C. pneumoniae* DNA was positive in 2 of 14 plaques (14.3%) and *H. pylori* was positive in 3 of 14 of plaques (21.4%) and one of 15 LIMA groups (6.7%) but the differences were not statistically significant ( $p=0.224$  and  $p=0.330$ , Fisher's test) (Figures 1 & 2). There was one patient in the plaque group in whom both *C. pneumoniae* and *H. pylori* DNA's were positive. Apolipoprotein B levels were significantly higher in *C. pneumoniae* IgM positive cases (0.844 g/L) compared with negatives (0.661 g/L) ( $p=0.004$ ,  $t=3.129$ ). Although IL-6 and hs-CRP levels were higher in both *C. pneumoniae* and *H. pylori* DNA positive subjects compared with negatives, it was not statistically significant (Tables 2 & 3). All DNA positive subjects were IgG positive for both bacteria and IgM positive for *C. pneumoniae*.

**Discussion.** The relation between *H. pylori* and atherosclerosis was determined as seropositivity in many studies, but there are only a few reports that detected *H. pylori* in atherosclerotic plaques<sup>11,12</sup>

Table 1 - Characteristics and inflammation parameters of patients in the atherosclerotic and control groups.

Parameter	Atherosclerotic group (n = 14)	Control group (n = 15)	p
Mean age ± SD	60.21 ± 11.03	59.73 ± 10.52	0.905
Gender (no. male / no. female)	9 / 5	11 / 4	0.700
Interleukin-6 levels (U/L)	36.53 ± 58.27	3.07 ± 8.25	0.052*
CRP levels (mg/L)	7.68 ± 14.79	16.99 ± 38.36	0.403
Apolipoprotein B levels (g/L)	0.70 ± 0.16	0.67 ± 0.10	0.549
<b>Patients with</b>			
Hypertension	7	7	0.858
Diabetes	4	3	0.682†
Hypercholesterolemia	4	3	0.682†
Smoking	9	9	0.812
Obesity	5	3	0.427†
* - Equal variances were not assumed by Levene's statistics, so p-value was calculated with corrections, † - Fisher's exact test was used because of small groups, CRP - C-reactive protein.			

Table 2 - Serological parameters of patients with respect to *Chlamydia pneumoniae* (*C. pneumoniae*) DNA positivity.

Parameter	<i>C. pneumoniae</i> DNA positive (n = 2)	<i>C. pneumoniae</i> DNA negative (n = 27)	p
<i>C. pneumoniae</i> IgM positivity	2	10	0.163
<i>C. pneumoniae</i> IgG positivity	2	25	1.000*
Interleukin-6 levels (U/L)	42.25 ± 59.75	17.52 ± 43.19	0.449†
Apolipoprotein B levels (g/L)	0.58 ± 0.07	0.70 ± 0.13	0.21
C-reactive protein levels (mg/L)	66.58 ± 93.94	8.49 ± 8.57	0.542†
* - Fisher's exact test was used because of small groups, † - Equal variances were not assumed by Levene's statistics, so p-values were calculated with corrections.			

Table 3 - Serological parameters of patients with respect to *Helicobacter pylori* (*H. pylori*) DNA positivity.

Parameter	<i>H. pylori</i> DNA positive (n = 4)	<i>H. pylori</i> DNA negative (n = 25)	p
<i>H. pylori</i> IgA positivity	1	4	0.553*
<i>H. pylori</i> IgG positivity	4	24	1.000*
Interleukin-6 levels (U/L)	56.37 ± 69.06	13.28 ± 36.79	0.303†
Apolipoprotein B levels (g/L)	0.71 ± 0.23	0.68 ± 0.12	0.733
C-reactive protein levels (mg/L)	21.93 ± 41.38	10.98 ± 27.78	0.498
* - Fisher's exact test was used because of small groups, † - Equal variances were not assumed by Levene's statistics, so p-values was calculated with corrections.			

whereas several studies failed to demonstrate any evidence of *H. pylori* in the atherosclerotic plaques of abdominal aortic aneurysms<sup>7</sup> and carotid atherosclerotic arteries.<sup>13</sup> There are also studies that could not determine any serologic relation between *H. pylori* and atherosclerosis.<sup>14</sup> The relationship between *C. pneumoniae* and atherosclerosis was first detected by Saikku et al.<sup>15</sup> In the following years, CAD risk was found to triple in *C. pneumoniae* seropositive cases compared with seronegatives.<sup>16</sup> These studies only showed serum antibody levels which could not differentiate acute or past infection, and in our country and region, as more than 90% of the population is seropositive for *C. pneumoniae*, infection, atherosclerosis relation has to be put forth with direct relation. In this study the atherosclerosis-bacteria relation was searched by direct identification of bacteria DNA in atherosclerotic plaques.

Blasi et al<sup>7</sup> found 51% *C. pneumoniae* DNA positivity in 51 atherosclerotic aortic plaques. There were a few studies reporting successful *C. pneumoniae* isolation from atherosclerotic plaques in cell cultures.<sup>17,18</sup> Farsak et al<sup>11</sup> studied *H. pylori* and *C. pneumoniae* and Prager et al<sup>9</sup> studied *C. pneumoniae* DNA in both atherosclerotic and normal vessels, and detected both microorganisms significantly higher in atherosclerotic vessels. There are also studies that could not find *C. pneumoniae* DNA in atherosclerotic plaques.<sup>20-26</sup> In our study, both *C. pneumoniae* and *H. pylori* DNA's were detected but normal and atherosclerotic groups did not differ significantly.

In this study, LIMA was chosen as the control group. The LIMA was examined macroscopically and arteries with plaques were not included in the study. In addition, none of the arteries' lumens were narrowed. There are studies that examined LIMA both histologically and radiologically. Peric et al<sup>27</sup> studied 80 LIMA's in randomly selected patients and could not find any angiographically significant atherosclerotic lesion. In the study of Kobayashi,<sup>28</sup> intima-media ratio (R) of 274 sections of 26 LIMA arteries were detected and the severity of atherosclerosis was graded from 1 to 4. In all of 274 IMA sections, grade 4 atherosclerosis was found in only one section, which meant that the whole artery segments of IMA's were homogenous without any atherosclerotic involvement.

We amplified DNA's of the bacteria using nested PCR technique, which was demonstrated to improve sensitivity in *H. pylori* detection.<sup>29</sup> Apfalter et al<sup>30</sup> compared 16 test methods for *C. pneumoniae* DNA detection in 9 centers by means of PCR. Positivity rates of atherosclerotic samples varied between 0-60% for the different test methods. The outer membrane protein-1 (OMP) region amplification with nested PCR technique and agarose gel electrophoresis, which we also used in this study,

had the best detection rates (60%).<sup>30</sup> In the study of Mahony et al,<sup>31</sup> all of the 11 positive *C. pneumoniae* DNA samples were detected by the same method used in the current study.

We determined DNA of the bacteria and assessed their relation with serum CRP and IL-6 concentrations, which proved to increase in atherosclerosis.<sup>2,32</sup> We detected higher levels of IL-6 and CRP in *C. pneumoniae* and *H. pylori* DNA positive subjects but yet not enough for statistical significance. C-reactive protein has been detected in early human atherosclerotic plaques and its appearance in plaques has been related to intima-media thickness of carotid arteries.<sup>33,34</sup> In the meta-analysis summarizing the results of 11 prospective studies with a total of 1953 cases, comparing patients in the bottom third of baseline measurements of CRP, patients in the top third had an odds ratio for CAD risk of 2.0 (95% confidence interval, 1.6-2.5) after adjustment of various confounders.<sup>35</sup> These findings support that increased CRP is a risk factor for stroke and cardiovascular events.

Recent in vitro studies have shown that *C. pneumoniae* infection of human smooth muscle cells resulted in production of IL-6, which is the major regulator of CRP production suggesting a plausible pathophysiological link.<sup>36,37</sup> In our study, both IL-6 and CRP levels were increased in DNA positive patients. These results show that the relation between *C. pneumoniae* infection and inflammation markers was not accidental, but rather supports infection hypothesis in atherosclerotic plaques. High CRP titers alone have been proven to be a risk factor for CAD and one of the etiologies for this increase might be chronic bacterial infections of the arterial walls. There are only a few studies comparing inflammation markers with DNA positivity. Johnston et al<sup>1</sup> and Prager et al<sup>19</sup> also found CRP levels higher in *C. pneumoniae* DNA positive patients whereas Berger et al<sup>38</sup> could not find any difference.

Results of many studies show that apo B is a better marker of risk of vascular diseases than any other lipid markers. In the review article of Sniderman et al,<sup>39</sup> the results of 4 large prospective studies have shown that apo B is superior to total cholesterol or low density lipoprotein (LDL) cholesterol to predict the risk of vascular diseases or predict future coronary events; and the ratio of apo B/apo A-1 is superior to total cholesterol/HDL cholesterol as an overall index risk. In the apolipoprotein-related mortality risk (AMORIS) study,<sup>40</sup> 175,553 Swedes were followed for an average of 5.5 years with the clinical event of fatal myocardial infarction and found that apo B was superior to LDL cholesterol in every direct comparison of patients in regard to age and gender of the patients. Haidari et al,<sup>41</sup> compared apo B

levels in 567 atherosclerotic patients diagnosed by coronary angiography and found a significant correlation between severity of atherosclerosis and serum apo B concentrations in the normolipidemic group.

To our knowledge there is no study comparing apo B with either *C. pneumoniae* or *H. pylori* DNA positivity. There is one study comparing seropositivity with apo B. Mendall et al<sup>42</sup> determined a positive relation between *H. pylori* and *C. pneumoniae* seropositivity with increased CRP levels and CRP concentration was associated with raised apo B, total cholesterol and triglyceride values. In our study, apo B serum levels and DNA positivity were not related but apo B levels were higher in *C. pneumoniae* IgM positive cases compared with negatives. We suggest that acute *C. pneumoniae* infection caused a change of the lipid profile to atherogenic profile as there was no change in apo B levels in chronic *C. pneumoniae* infection. In the study of Laurila et al,<sup>43</sup> serum triglyceride and total cholesterol concentrations were significantly higher in patients positive for IgG and IgA antibody for *H. pylori* compared with negatives. The previous studies, and this study, support the hypothesis that infections modify the serum lipid profile in a way that increases the risk of atherosclerosis.

In conclusion, *C. pneumoniae* and *H. pylori* DNA were not statistically significantly higher in atherosclerotic plaques compared with non-atherosclerotic control arteries. In DNA positive subjects, although not statistically significant, high titers of CRP and IL-6 were detected, which proved to increase in atherosclerosis. The increased titers of inflammation markers in DNA positive cases may be related to infections in atherosclerotic plaques, however, the results should be reproduced in a larger cohort.

**Acknowledgments.** This research has been granted by government budget from the National Organization Institute Extensive Research Projects for Universities in Turkey. Project No: 002 K 120590.

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