ORIGINAL REPORT

Chlamydia pneumoniae in the Atherosclerotic Plaques of Coronary Artery Disease Patients

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Abstract- An association between *Chlamydia pneumoniae* (C. *pneumoniae*) and cardiovascular disease has been demonstrated. In this study, we aimed to study this potential relationship in 105 Iranian patients. Coronary artery specimens from 105 Iranian patients undergoing CABG were analyzed by PCR method for C. *pneumoniae*. Serological evaluation for C. *pneumoniae* IgG and IgM was performed using ELISA. 53 specimens from mamillary artery were also investigated. C. *pneumoniae* PCR test result was positive for 23 (21.9%) of patients with coronary artery atherosclerosis, but none of the specimens from the mamillary artery was positive for C. *pneumoniae* when it was evaluated by the PCR (P<0.001). Coronary artery disease patients with and without a history of unstable angina or myocardial infarction were comparable in C. *pneumoniae* PCR test positive rates (P=0.618). Relevance of IgG and IgM positivity were also studied by correlating it to the study parameters, but no difference was found. CRP was significantly higher in the IgM positive group (P<0.001). A significant proportion of coronary atherosclerotic plaques are infected with C. pneumoniae while no infection was found in the normal mamillary artery specimens. No association was found between acute coronary syndromes and serological and PCR positivity. Further prospective randomized controlled studies with large patient population are needed to confirm our findings.

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Keywords: Atherosclerosis; Chlamydia pneumonia; Coronary heart disease; Infection

Introduction

Atherosclerosis is the foremost cause of coronary vascular disease (CVD), which is the leading cause of death in the industrialized world as well as a major accountable due for mortality in the developing countries, and it also induces a significant burden of morbidity in the entire world. The process which results in the atherosclerotic lesions initiates as a protective reaction to insults happening to the arterial wall components including the endothelium and smooth muscle cells, and then it advances to the formation of permanent atherosclerotic plaques which narrow and even thoroughly obstructive to the artery lumen.

Nowadays, atherosclerosis is recognized as a consequence of some types of chronic inflammatory processes in which macrophages play major roles; and growing evidence empowers the concept that infection may be one of these disease processes inducing inflammation leading to cardiovascular disorders. In recent years, attention has increasingly focused on the role of infection because the general belief is that an infective process can be prevented by using antibiotics (which needs to be proved through experimentation); and the frequent respiratory pathogen *Chlamydia pneumoniae (C. pneumoniae),* an obligate intracellular Gram-negative bacteria, has attracted the highest rate of suspicion as a cardiovascular risk factor (1).

C. pneumoniae was initially isolated from the conjunctiva of a child participating in a trachoma

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vaccine trial in 1965, nevertheless, up until 1986; it was not officially established as a distinct species of *Chlamydia* (2,3). *C. pneumoniae* infections are often asymptomatic or associated with mild illness of upper respiratory tract. Nevertheless, several reports have linked some chronic and serious illnesses to the presence of *C. pneumoniae* including chronic bronchiolitis (2,3) chronic otitis media and chronic persistent pharyngitis (4,5), asthma (6). However, respiratory diseases are not the only disorders which have been associated with *C. pneumoniae* infections among which reactive arthritis and neurodegenerative diseases are notable (7,8).

The evidence for a real association between *C. pneumoniae* and atherosclerosis is quite more investigated than that for other infectious agents. Several studies have pointed to a role for *C. pneumoniae* in the development of atherosclerosis by using seroepidemiological, histological and experimental examinations (9). However, the main methods through which these investigations were performed can be divided into two groups: serological assessment (10-13), and techniques demonstrating the presence of the organism in atherosclerotic plaques including PCR, electron microscopy, ELISA, and tissue culture (14-17). The chronic persistence of infection in CVD patients has also been shown using demonstration of circulating *C. pneumoniae*-specific immune complexes in a high proportion of the patients (18). In this study, we aimed to study any potential associations between *C. pneumoniae* detected by different methods and coronary arterial atherosclerosis in a large patient population in Iran.

Materials and Methods

The study included 105 consecutive patients who were admitted to the Department of Cardiovascular Surgery of Baqiyatallah University of Medical Sciences hospitals between 2008 and 2010 with various manifestations of ischemic vascular disease, and who underwent coronary artery bypass grafting (CABG) surgical interventions there. In addition, 53 specimens from biopsies of macroscopically healthy regions of the left internal mamillary artery in patients who had undergone CABG were collected at the National Forensic Medicine Department. Data on demographics, smoking habit, lipid profile, and medical history were recorded for all subjects. Acute coronary syndrome was defined as myocardial infarction and/or unstable angina.

This study was approved by the University Research Review Board (URRB) and the Ethics Committee of Baqiyatallah University of Medical Sciences. All subjects provided written informed consent to participate in the study and were assured that their personal information will remain anonymous and confidential.

Tissue samples were dissected in the operating room and stored under sterile conditions. Artery segments were placed in microcentrifuge tubes using binding buffer. Transport vials were sealed in the operating room and opened only in the laminar airflow safety cabinet at the microbiology laboratory. All of the specimens were kept at -20º until processing. For preparation of genomic DNA and PCR, DNA was extracted from endoarterectomy specimens by using the QIAamp tissue mini-kit (Qiagen Inc, Valencia, CA, USA). The DNA absorbed in the QIAamp spin column was eluted with 55µL of Tris EDTA and then subjected to the PCR.

For preparation of genomic DNA and PCR, DNA was extracted from endoarterectomy specimens by using the QIAamp tissue extraction kit according to kit manufacturer procedure. PCR was carried out for *C.* pneumoniae using primers, HL1 (GTTGTTCATGAAGGCCTACT-3´) and HR1 (5´-TGCATAACCTACGGTGTGTT-3´), which amplify a 437-bp fragment of the PstI cloned gene (19). PCR products were visualized by electrophoresis by 1.5% agarose gel, stained with ethidium bromide, and examined under UV illumination. Serological evaluation for *C. pneumoniae* IgG and IgM was performed using ELISA.

Statistical analysis

Data was analyzed using SPSS software version 17.0 (SPSS Corp., Chicago, IL, USA). Chi square test, Fisure's exact test, Student's t test, Mann-Whitney U test and Kruskal-Wallis test were used where appropriate. Kolmogorov-Smirnov test was used for evaluating data distribution pattern. All statistical analyses were performed at the 0.05 significance level.

Results

Characteristics of the study participants are summarized in table 1. Data of overall 105 patients and their biopsy specimen were entered into the analysis. *C. pneumoniae* PCR test result was positive for 23 (21.9%) of patients with coronary artery atherosclerosis. Serologic test results also showed only 5 (4.8%) positive case for *C. pneumoniae* IgM, but 48 (45.7%) for *C. pneumoniae* IgG tests.

Table 1. characteristics of the study participants.

Parameters	Result
Mean age ± SD (yr)	58.2±10.6
Gender male (%)	73 (69.5)
Mean weight ± SD (Kg)	76.7±10.6
Mean BMI ± SD (Kg/m^2)	28.1±4.0
IgG *C. pneumoniae*	90 (85.7)
IgM *C. Pneumoniae*	4 (3.8)
PCR *C. pneumoniae*	28 (26.7)
Biochemical examinations	
Triglyceride (mean±SD)	195.3±110.4
Fasting blood glucose (mean±SD)	150.9±67.2
Fibrinogen (mean±SD)	203.8±54.8
Cholesterol total (mean±SD)	196.7±189.7
LDL cholesterol(mean±SD)	97.1±38.5
HDL cholesterol (mean±SD)	42.2±11.1
CRP(mean±SD)	1.7±0.46
Medical history	
Hypertension	57 (54.3)
Smoking	26 (24.8)
Acute coronary syndromes	62 (59)
Diabetes mellitus	54 (51.4)

28 (26.7%) of patients had a history of unstable angina or myocardial infarction. Coronary artery disease patients with and without a history of unstable angina or myocardial infarction were comparable in *C. pneumoniae* PCR test positive rate (6 (26.1%) vs. 17 (73.9%), respectively; *P*=0.618). We also correlated PCR test results for *C. pneumoniae* with family history for cardiovascular diseases; but found no statistical difference (Table 2).

We also reanalyzed data to find out whether *C. pneumoniae* replication in the atherosclerotic plaques have any predictors. For this purpose, we correlated demographic and medical history of patients with their *C. pneumoniae* PCR test results. We found no difference between the two patient groups regarding any of the parameters (Table 2).

Relevance of IgG positivity was studied by correlating it to the study parameters, but no difference was found: family history (*P*=1.0), diabetes mellitus (*P*=0.241), history of arterial hypertension (*P*=0.245), acute coronary syndrome (*P*=0.833), fasting blood glucose (*P*=0.722), fibrinogen (*P*=0.501), triglyceride (0.612), cholesterol (*P*=0.666), LDL (*P*=0.743), HDL (*P*=0.095), CRP (*P*=0.424). Older patients were significantly more likely to be *C. pneumoniae* IgG positive (*P*=0.033).

C. pneumoniae IgM was only found in 5 (4.8%) of atherosclerotic patients. We also correlated *C. pneumoniae* IgM test result with the study parameters; but found no relationship unless for CRP which was significantly higher in the IgM positive group (1.7±0.4 vs. 2.0±0.0; *P*<0.001).

Then we reanalyzed data for coronary arterial atherosclerotic lesions and compared them to that of 53 mamillary artery specimens. None of the specimens from the mamillary artery was positive for *C. pneumoniae* when it was evaluated by the PCR which was significantly lower than that for the coronary artery plaques (*P*<0.001).

Table 2. Comparison of the study participants' characteristics regarding their serological analysis and their atherosclerotic plaques' PCR test results for *C. pneumoniae*.

Parameters	*C. pneumoniae* positive subjects by IgG			*C. pneumoniae* positive lesions by PCR		
	IgG positive	IgG negative	*P* value	PCR positive	PCR negative	*P* value
Mean age ± SD	62.7±9	58.7±9.8	0.033	62.9±10.4	59.9±9.4	0.191
Gender male (%)	38 (66.7)	35 (72.9)	0.529	16 (69.6)	57 (69.5)	1.0
Mean weight ± SD (Kg)	77.9±10.9	74.3±9.5	0.071	78.5±9.5	75.3±10.5	0.186
Mean BMI ± SD (Kg/m^2)	27.9±3.7	27.4±3.1	0.473	27.8±2.9	27.6±3.5	0.83
Biochemical examinations						
Triglyceride (mean±SD)	205.5±131.3	186.6±95.4	0.475	165.4±79.0	199.3±115.3	0.353
Fasting blood glucose (mean±SD)	147.3±59.4	156.6±76.4	0.532	1385±81.8	155.1±66.8	0.412
Fibrinogen (mean±SD)	199.3±49.8	205.8±58.5	0.623	183.6±56.9	209.1±53.2	0.094
Cholesterol total (mean±SD)	177±47.7	179.2±35.8	0.827	172.2±41.2	179.4±40.7	0.592
LDL cholesterol(mean±SD)	101±41.2	98.2±36.9	0.767	102±30.6	98.8±39.6	0.81
HDL cholesterol (mean±SD)	39.9±8.8	44.3±12.3	0.078	41.5±11.9	42.8±11.2	0.742
CRP(mean±SD)	1.0±0.4	1.8±0.4	0.846	1.9±0.3	1.7±0.4	0.120
Medical history						
Family history for CVD	9 (15.8)	186.6±95.4	1.0	165.4±79.0	1 (4.3)	0.111
Hypertension	23 (47.9)	156.6±76.4	0.245	1385±81.8	46 (56.1)	0.490
Smoking	12 (21.1)	205.8±58.5	0.371	183.6±56.9	22 (26.8)	0.424
Acute coronary syndromes	17 (29.8)	179.2±35.8	0.833	172.2±41.2	27 (32.9)	0.618
Diabetes mellitus	26 (45.6)	98.2±36.9	0.241	102±30.6	43 (52.4)	0.814

Discussion

The first reports on the presence of an etiological association between chlamydia species and cardiovascular diseases were about *Chlamydia – trachomatis* and - *psiittaci* induced myocarditis and endocarditis (20,21). Subsequently some investigators focused on cardiovascular disorders and their potential associations with *C. pneumoniae* (22). What makes research on any association between infection with this pathogen and cardiovascular diseases more crucial is the very high prevalence of infection with *C. pneumoniae* in the general population as well as the prevalence, mortality and morbidity that these disorders compel on human societies. The prevalence of antibodies to *C. pneumoniae* reaches to 50% in young adults throughout the world, with a 25% higher prevalence in men than in women, which can mirror the higher prevalence of CVD in males (23).

Saikku *et al.* (24) were one of the premiere scientists who started investigations on a possible association between *C. pneumoniae* and coronary artery disease in 1988. In this study, authors analyzed blood samples from patients with coronary disorders (chronic disease and myocardial infarction) and a normal control group for antibodies to *C. pneumoniae*. Patients with myocardial infarction and chronic coronary disease represented significantly higher levels of IgG or IgA antibody levels to the *C. pneumoniae* antigen than those in the control group. Based on these findings, Saikku *et al.* concluded that chronic *C. pneumoniae* infection might have an etiological association with coronary artery disease. Similar results have been reported by Maia *et al.* (25) who observed an association between the serum titers of anti-Chlamydia antibodies in the acute phase of patients with unstable angina or myocardial infarction.

Maybe the strongest data on a potential association between *C. pneumoniae* and CVD came from a population based randomized controlled trial (26). Four thousand patients enrolled in the 5 year Helsinki Heart Study and finally it was revealed that patients who had coronary events more frequently exhibited serologic evidence of chronic *C. pneumoniae* infection (elevated IgA and lipopolysaccharide immune complex levels) compared to other study subjects. Pesonen *et al.* (27) also found that chlamydial lipopolysaccharide is a prognostic factor for a prospective cardiovascular event. A recent study by Petyaev *et al.* (28) investigated serum samples of 56 patients attending their center with an acute coronary syndrome episode for the presence of *C. pneumoniae* in the circulating blood using PCR, and compared them with 26 healthy volunteers. In their study, 21% of patients with acute coronary syndrome represented positive PCR results for *C. pneumoniae* which was significantly higher than that in the healthy volunteers (7.7%). Using almost the same methodology but on patients with abnormal angiogram (not acute disease), West *et al.* (29) found no positive PCR result for case or control group. Sawayama *et al.* (30) and Pesonen *et al.* (31) observed that high *C. pneumoniae* IgA antibody titer is associated with an increased risk of atherosclerotic diseases; although the latter investigated carotid atherosclerosis plaques. Satpathy *et al.* reported that despite a higher rate of anti *C. pneumonial* antibodies found in coronary artery patients, they found atherosclerotic plaque specimen with a positive report on PCR analysis for detection of *C. pneumonia*. In the current study, however, we found no association between coronary events and *C. pneumoniae* IgG and IgM and PCR test results. This disparity might be related to the detection technique while we have not evaluated IgA and lipopolysaccharide immune complex levels and they did not analyze atherosclerotic specimens by PCR.

A significant study on the possibility of presence of an association between atherosclerosis and *C. pneumoniae* infection was performed by Kuo *et al.* (17) who enrolled autopsy specimens from young adults. They found that none of the subjects with normal coronary arteries represented the presence of *C. pneumoniae* detected by PCR method while 86% of subjects with atheroma had a positive PCR test for *C. pneumoniae*. Authors finally suggested that coronary lesions in young adults with atherosclerosis may be associated with prior *C pneumoniae* infection. Another study also showed that patients who had undergone CABG had a significantly higher rate of *C. pneumonial* presence in the arterial specimens detected by PCR when compared to healthy control autopsies (32). This finding is consistent with our study results; we did not find any positive PCR tests for the normal mamillary arteries which was significantly lower than that in the coronary atherosclerotic plaques. Nevertheless, one may declare that because normal controls in our study were from the mamillary artery specimens, we may not compare them with coronary arterial lesions. Although this is a logical statement, however, putting our study findings together with those of the Kuo *et al.*, we may be able to say that the natural resistance of mamillary artery towards atherosclerosis may be associated with its immunity to *C. pneumonial* infection. This conclusion

more significantly comes into view when we emphasize that all these specimens including those of the mamillary artery were retrieved from the same patients with coronary atherosclerosis.

Alongside the evidence towards a positive relationship between *C. pneumoniae* and atherosclerosis formation in the coronary arteries, some other surveys doubted this issue. In a recent study by Hoymans *et al.* (33), authors investigated the blood circulation of 151 consecutive patients with ischemic heart disease for antibodies against human and Chlamydia HSP60 and *C. pneumoniae* IgG and correlated to the angiographic extent of coronary atherosclerosis, with clinical symptoms of ischemic heart disease and with biochemical and functional endothelial dysfunction markers. They found no association between antibody responses to *C. pneumoniae* IgG, human or Chlamydia HSP60 and endothelial dysfunction and presence and severity of coronary artery disease, arguing against the suggestion that infection contributes to disease progression and suggesting that *C. pneumoniae* is an unlikely major risk factor of coronary atherosclerosis.

The pathogenesis of *C. pneumoniae*-induced atherosclerosis is of outmost relevance, because knowing this/these mechanisms; we would be able to develop preventive strategies for atherosclerogenic action of *C. pneumoniae*. Several studies have been conducted concerning the mechanism through which *C. pneumoniae* induces its ominous effects on the atherosclerosis. One of the most investigated mechanisms is through cytokines. Campbell *et al.* (34) showed statistically significant increases in the plasma levels of IL-2, IL-5, IL-6, IL-10, IL-12, GM-CSF, IFN-gamma, and serum amyloid A after infection with *C. pneumoniae.* There was also a decrease in the activity of the HDL protective enzyme paraoxonase as well as a reduced ability of HDL to prevent oxidation of palmitoyl-2-arachidonyl-sn-glycerol-3-phosphocholine by hydroperoxyoctadecadienoic acid at 48 and 72 h post-infection. They also investigated a potential impact of *C. pneumoniae* induced acute phase response on atherosclerotic plaque stability with measuring the frequency of intra-plaque hemorrhage as a marker of plaque disruption in mice. There was an increased frequency of intra-plaque hemorrhage only in the older mice infected with the live organisms (and not killed ones). Based on their findings, authors suggested that acute phase reactant proteins produced in response to pulmonary infection with *C. pneumoniae* may contribute to the progression and destabilization of atherosclerotic lesions. Sessa *et al.* (35) suggested a provocative role for the mentioned microorganism for TNF-alpha and Mousa *et al.* (36) found this association with IL-18. A potential association between *C. pneumoniae* and cholesterol metabolism has also been proposed by Liu *et al.* (37) who in their *in vitro* survey showed that both live and inactivated *C. pneumoniae* infection induce intracellular cholesterol accumulation and foam cell formation - a hallmark of early atherosclerosis. An in vivo study by Chen *et al.* (38.) showed that *C. pneumoniae* infection is associated with a significant increase in size and lipid content of the atherosclerotic lesions in mice. Furthermore, *C. pneumoniae* infection was associated with significant increases in serum concentrations of inflammatory cytokines and numbers of macrophages in their plaques. On the other hand, mice that had not genes of some types of cytokines developed significantly less acceleration of lesion size following *C. pneumoniae* infection compared with controls despite similar levels of blood cholesterol levels. Their data suggests that *C. pneumoniae* can affect genetically more susceptible mice, and one may generalize this concept to humankind, as well. In the current study, due to the methodology employed, we could have no mention about the atherosclerogenic mechanism of *C. pneumoniae.* Deniset *et al.* (39) in an ex-vivo study showed that *C.* pneumonia infection can stimulate arterial thickening in a complex vessel environment without the presence of a host immune response. It also has been suggested that *C. pneumoniae* is associated with metabolic syndrome (40).

Some studies have also concentrated on the potential impact of antibiotic therapy on the cardiovascular effects of *C. pneumoniae*. Stone *et al.* (41) investigated 325 patients admitted with acute myocardial infarction or unstable angina (acute coronary syndromes)for a potential impact of anti-helicobacter and *C. pneumoniae* therapy on the cardiac outcome. Study subjects were randomized to receive a 1-week course of 1 of 3 treatment regimens: (1) placebo; (2) amoxicillin (500 mg twice daily), metronidazole (400 mg twice daily), and omeprazole (20 mg twice daily); or (3) azithromycin (500 mg once daily), metronidazole (400 mg twice daily), and omeprazole (20 mg twice daily) and were followed for 1 year. CRP levels were reduced in unstable angina patients receiving amoxicillin, and fibrinogen was reduced in both patient groups receiving antibiotics. No difference in frequency or timing of Cardiac death and readmission with acute coronary syndrome was observed between the 2 antibiotic groups. At 12, weeks, there was a 36% reduction in all end points in patients receiving antibiotics compared with

placebo which was statistically significant (P=0.02). This reduction persisted during the 1-year follow-up. This study suggests that even a 1 week antibiotic therapy against helicobacter pylori and *C. pneumoniae* can reduce heart events, but does not differ between the two pathogens. In conclusion, the current study showed that a significant proportion of coronary atherosclerotic plaques are infected with *C. pneumoniae* while no infection was found in the normal mamillary artery specimens. No association was found between acute coronary syndromes and serological and PCR positivity. Further prospective randomized controlled studies with large patient population are needed to confirm our findings.

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