Original Article

Acute and chronic *Chlamydia pneumoniae* infection and inflammatory markers in coronary artery disease patients

Mehvash Haider[1], Meher Rizvi[1], Abida Malik[1], Mohammad Azam[1], Mohammad U. Rabbani[2]

Department of Microbiology[1] and Department of Medicine[2], Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, 202001, India

Abstract

Introduction: We evaluated the role of inflammation and acute or persistent *Chlamydia pneumoniae* infection in coronary artery disease (CAD).

Methodology: The study involved 63 cardiovascular disease patients diagnosed with angina and myocardial infarction (MI) and 40 healthy controls. ELISA was performed for detection of *C. pneumoniae* IgA antibodies and for quantitative analysis of IFN-*γ*. PCR was performed for detection of the *C. pneumoniae* 16 SrRNA gene in blood.

Results: *C. pneumoniae* IgA antibodies were detected in 66.66% cases and 41.37% controls. Of IgA seropositive cases 71.43% were MI patients, 61.90% were stable angina patients, and 64.29% unstable angina patients. Of 40 patients whose PCR was done 32.5% were positive of which 76.92% were IgA seropositive. Traditional risk factors were not significantly associated with CAD. The mean value of IFN-γ in cases was 32.12pg/ml and 11.32pg/ml in controls. Elevated IFN-γ was observed in 76.92% of *C. pneumoniae* IgA seropositives with a mean value of IFN-γ in angina patients of 3.39pg/ml, in unstable angina of 12.91 pg/ml and in MI patients of 23.89 pg/ml. IFN-γ levels in cases who were positive for *C. pneumoniae* infection by serology and PCR was 55.21 pg/ml.

Conclusion: *C. pneumoniae* infection was significantly associated with CAD risk. The role of acute or persistent infection in progression of CAD to adverse clinical outcome was evident by a high percentage of seropositives among PCR positives. Although IFN-γ alone had a role to play in development of CAD, its values were further enhanced due to recurrent *C. pneumoniae* infection.

Key words: *Chlamydia pneumoniae*; coronary disease (CAD); Interferon-γ; serology; PCR

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Introduction

Cardiovascular disease (CVD) is now the leading cause of death, accounting for 29% of all deaths in 2005, according to the World Health Organization (WHO) [1]**.** It has been reported that Indians have the highest risk of coronary artery disease (CAD) and the prevalence of CAD in India has recently been estimated to be 11% in the middle-aged group. Despite advances in preventive medicine and controlling of risk factors, CVD is still a major public health problem. The known risk factors of cardiovascular disease are not sufficient to explain all the epidemiological variables and fluctuations of the disease. These observations fueled renewed interest in a link between CVD and other possible risk factors.

The hypothesis of infectious etiology of atherosclerosis is one of the most interesting areas of vascular research. Various pathogens such as herpes viruses [2]**,** CMV [3]**,** *Heliobacter pylori* [4]**,** *Streptococcus mutans*, *Porphyromonas gingivalis*, *ActinobacilIus actinomycetemcomitans* and *Prevotella intermedia* [5] at one time or another have been implicated in causing CVD. This entire infectious process may involve pro-inflammatory mechanisms such as interleukins and cytokines.

The first suggestion that *Chlamydia pneumoniae* may be associated with atherosclerosis was proposed in 1988 by Saikku *et al*. [6]; however, whether it actually plays a role in causing CAD is still questionable. Since atherosclerosis is a chronic inflammatory process [7], infections, especially those caused by *C. pneumonia* [6,8], have been suggested to play a role through immune activation. We examined the relationships between acute and persistent *C. pneumonia* infection and the level of inflammatory activity in patients with coronary artery disease. We hypothesized that *C. pneumonia* infection in these patients could lead to increased inflammatory response.

Methodology

A cross-sectional study with a control arm was conducted at the Departments of Microbiology and Medicine, Jawaharlal Medical College, AMU, Aligarh, India, from November 2008 to September 2009. The study group consisted of 63 randomly selected patients with coronary artery disease. The control arm consisted of 40 age- and sex- matched healthy volunteers. Controls were confirmed for absence of disease by measurement of blood pressure and relevant investigations. The case to control ratio was 1:5. The study approach was three-pronged: persistent infection of *C. pneumonia* was assessed by the level of IgA antibodies to *C. pneumonia*; acute infection of *C. pneumonia* was assessed by the detection of the SrRNA gene of *C. pneumoniae*, and the level of interferon gamma (IFN-γ) in serum was used to assess inflammatory activity.

We included only patients with CAD. CAD is defined as a progressive reduction in blood flow to the myocardium due to the buildup of atheromatous plaques. It manifests as stable angina, unstable angina, and myocardial infarction. Unstable angina is defined as one of the following in presence of chest pain occurring at rest that lasts for longer than 10 minutes, or chest pain of severe and new onset (within the prior four to six weeks), or chest pain occurring with a crescendo pattern (more severe and more frequent). Stable angina is defined as chest pain which begins slowly and gets worse with physical activity and is relieved on medication or rest. Myocardial infarction is defined as chest pain lasting more than 30 minutes with ST segment elevation/depression, evolving Q waves, symmetric inversion of T waves, and elevation of cardiac markers. Patients were confirmed as having coronary artery disease by testing for cardiac injury markers such as creatinine phosphokinase, Troponin-T, Troponin-I, coronary angiography, and stress testing. Exclusion criteria were as follows: patients with secondary hypertension (renal causes, endocrine causes, drug induced); patients with pulmonary involvement (e.g. chronic obstructive pulmonary disease [COPD], pneumonia, etc.) or with any other septic foci; and patients with history of antibiotic intake in the previous one month.

After obtaining informed consent, a detailed clinical history and medical examination was performed. Blood pressure was measured for all subjects in a quiet room using a sphygmomanometer and according to standard methods. A blood pressure reading higher than 140/90mm Hg on two or more occasions measured at least eight hours apart was considered as hypertension.

A sample of venous blood was also collected from each subject in the study after a 10-hour overnight fast for determination of their fasting total cholesterol and blood sugar using the enzymatic colorimetric method. Hypercholesterolemia was defined as a total cholesterol level of ≥ 6.2mmol/L. Blood sugar estimation was also performed in all patients and non-diabetic subjects were given 75 g of glucose after an overnight fast for an oral glucose tolerance test. A case of diabetes mellitus was defined as a subject with a known history or with a two-hour plasma glucose concentration ≥ 11.1mmol/L by the oral glucose tolerance test. Electrocardiograph (ECG) was performed in all cases. A non-smoker was defined as one who never smoked before, or who smoked cigarettes but not every day, or who smoked too little in the past to be regarded as an ex-smoker. A smoker included those who had a present history of smoking cigarettes daily or who was formerly smoking daily but had since given up the habit completely.

A written and informed consent was obtained from all subjects. Ethical clearance was obtained by ethical committee of JNMC.

Collection of sample for serology and amplification of 16 SrRNA

Taking all aseptic precautions, 10 ml blood was obtained by venepuncture, 5 ml in an EDTA vial and 5 ml in plain vial. Serum was separated from the samples in a plain vials by centrifugation and stored at -20ºC. Samples in the EDTA vials were used for DNA extraction by the phenol chloroform extraction method.

Amplification

PCR for detection of the 16 SrRNA gene of *C. pneumoniae* was performed for 40 cases and 11 controls. Primers were ordered from Fermentas, Life Sciences, Maryland, USA. The sequences for primer pairs of 16SrRNA were taken from published literature [10, 11, 12] as follows: 16SrRNA 5' GCT GGC GGC GTG GAT G 3' 832 bp 5' CGA CAC GGA TGG GGT TG 3' using Master Mix from Fermentas, Life sciences, Maryland USA.

Table 1. Socio-demographic and clinical characteristics of patients and controls

Variables	Patients (%) n = 63	Controls (%) n = 40
Age (years)		
≤ 19	-	-
20-29	-	-
30-39	5 (7.93%)	2 (5%)
40-49	22 (34.92%)	14 (35%)
50-59	23 (36.50%)	17 (42.5%)
≥ 60	13 (20.63%)	7 (17.5%)
Mean (± SD)	52.7 ± 8.9	54.0 ± 8.1
Gender		
Male	38 (60.32%)	25 (62.5%)
Female	25 (39.68%)	15 (37.5%)
Male/Female	1.5	1.7
Diagnosis		
Stable angina	21 (33.33%)	-
Unstable angina	14 (22.22%)	-
Myocardial infarction	28 (44.44%)	-

The reaction mixture consisted of 25 µl of Primer (Forward) 2µl, Primer (Reverse) 2µl, DNA 2µl, Nuclease free water 9µl, MasterMix 12.5µl. Cycling parameters for PCR were 94°C for 10 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 53°C for 50 seconds, elongation at 72°C for one minute and a final extension at 72°C for 7 minutes.

Serology

IgA antibodies to *Chlamydia pneumoniae* were detected by Sero CP IgA (Savyon Diagnostics, St. Ashdod, Israel).

The Human IFN-γ ELISA was used for the *in-vitro* quantitative determination of interferon gamma (IFN-γ) in human serum. The kit was obtained from Diaclone, Stamford, Connecticut, USA.

Statistical analysis

The data were entered and analysed using the Statistical Package for the Social Sciences version 17.0 (SPSS, Chicago, USA). Continuous variables were presented using mean ± standard deviation while categorical variables were presented as proportions. Categorical comparisons were made using Chi-square/Kruskal-Wallis test where appropriate and a P value of less than 0.05 was considered significant.

Results

Out of 63 cases, the majority of patients 45 (71.43%) were in the age group 40 to 60 years with maximum clustering (23; 36.50%) in the age group 50-59 years followed by 22 (34.92%) in the age group 40-49 years (Table 1). The mean age distribution was 52.7 ± 8.9 years in cases and 54.01 ± 8.1 years in controls. There were more males than females ($p < 0.01$), and significantly more males than females were angina patients ($p < 0.01$) and myocardial infarction (MI) patients ($p < 0.05$).

Distribution of traditional risk factors for development of CAD

Out of 63 cases, 19 (30.15%) were smokers, 5 (7.81%) were diabetics, 20 (31.75%) were obese, 12 (19.05%) had family history of cardiac problems, and 35 (55.55%) had past history of a cardiac episode (Table 2). None of the traditional risk factors were significantly associated with CAD.

Among the 19 smokers, 10 (52.63%) had myocardial infarction and six (31.58%) had angina.

Among the studied population, 20 (31.75%) were obese, of whom 13 (65%) had myocardial infarction and seven (35%) had angina.

Only 12 (19.05%) patients had family history of cardiac problems. Family history was present in nine (75%) MI patients and three (25%) angina patients. Overall, family history was insignificant in patients with cardiovascular diseases.

Among the studied cases, 35 (55.55%) had past history of a cardiac episode, most of whom were MI patients (18; 51.43%). As a whole, past history of a cardiac episode was significant ($p < 0.05$) in relation to cases.

Table 2. Gender distribution of CAD and traditional risk factors amongst patients

Variable	Male (%) (n1)	Female (%) (n2)	Total proportion (%) n1 + n2 / 63
Risk Factors			
Smoking	19 (100%)	-	19 (30.15%)
Diabetes mellitus	3 (60%)	2 (40%)	5 (7.81%)
Obesity	10 (50%)	10 (50%)	20 (31.75%)
Past History	20 (57.14%)	15 (42.86%)	35 (55.55%)
Family History	6 (50%)	6 (50%)	12 (19.05%)
Hypercholesterolemia	9 (50%)	9 (50%)	18 (28.57%)
Diagnosis			
Stable angina	8 (38.09%)	13 (61.90%)	21 (33.33%)
Unstable angina	11 (78.57%)	3 (21.43%)	14 (22.22%)
Myocardial infarction	19 (67.86%)	9 (32.14%)	28 (44.44%)

Assessment of infection and traditional risk factors for development of CAD

Of 42 IgA seropositive cases, 23 (54.8%) had past history, 14 (33.33%) were smokers, 10 (23.8%) had deranged lipid profiles, nine (21.4%) were obese, seven (16.1%) had family history, and five (11.91%) had diabetes. Of the 13 PCR positive cases, seven (53.85%) were smokers, four (30.76%) were obese, two (15.38%) had past history, one (7.69%) had family history, one had a deranged lipid profile and none had diabetes. Traditional risk factors were not strongly associated with PCR positivity on patients of cardiovascular disease.

However, when *C. pneumoniae* infection (serology in 66.66% cases, PCR in 32.5%) was considered, it was seen to be more significantly associated with CAD as compared to traditional risk factors (Table 3).

Detection of IgA antibodies to C. pneumoniae

C. pneumoniae IgA antibodies were detected in 42 (66.66%) cases and 12 (41.37%) controls. This test had a sensitivity of 66.66%, specificity of 51.72%, positive predictive value of 63.63%, negative predictive value of 41.67%, and an odds ratio of 2:14. Detection of cases was statistically significant ($p < 0.05$). Twenty (71.43%) of the MI patients were IgA positive, followed by 13 (61.90%) stable angina and 9 (64.29%) unstable angina patients. Among *C. pneumoniae* IgA seropositives, angina patients had sensitivity of 64.29%, specificity of 58.62%, positive predictive value of 42.86%, negative predictive value of 77.27%, and an odds ratio of 2:55. In *C. pneumoniae* IgA seropositive patients, there was MI sensitivity of 71.43%, specificity of 58.62%, positive predictive value of 56.25%, negative predictive value of 68% and an odds ratio of 3.54.

16 SrRNA gene of C. pneumoniae

PCR for detection of *C. pneumoniae* was performed for 40 cases and 11 controls. The majority (61.54%) of PCR positive cases were detected in the winter months of January and February as compared to 38.46% positive cases in March, April and July (Figure 1). Thirteen (32.5%) of the cases and none of the controls were positive on PCR. Of 40 patients whose PCR was done, 26 (65%) were *C. pneumoniae* IgA positive. Ten out of 26 (38.5%) patients of MI and three out of 26 (11.53%) angina patients were PCR positive (Figure 2). Among the 13 PCR positives, the majority (10; 76.92%) were *C. pneumoniae* IgA.

Estimation of IFN-γ levels

IFN-γ was raised in most of the patients with CVD. Values ranged from 8 pg/ml to 638 pg/ml. The mean level of Interferon-γ in these patients was 32.13pg/ml and that of controls was 11.32pg/ml (7-17 pg/ml). Forty-two (66.66%) of these cases were *C. pneumoniae* IgA seropositive. The mean level of Interferon-γ in stable angina patients was 3.39 pg/ml, in patients with unstable angina the level was 12.91 pg/ml, and in MI patients the level was 23.89 pg/ml. Among *C. pneumoniae* IgA seropositives, the mean value of INF-γ in stable angina patients was 4.33 pg/ml, 18.5 pg/ml in unstable angina patients, and 27.29 pg/ml in MI patients.

Figure 1. PCR positivity - Seasonal variations. PCR was performed on 40 cases of which 13 (32.5%) were positive. Of these positive cases 12 (92.3%) were detected in the months of January to April.

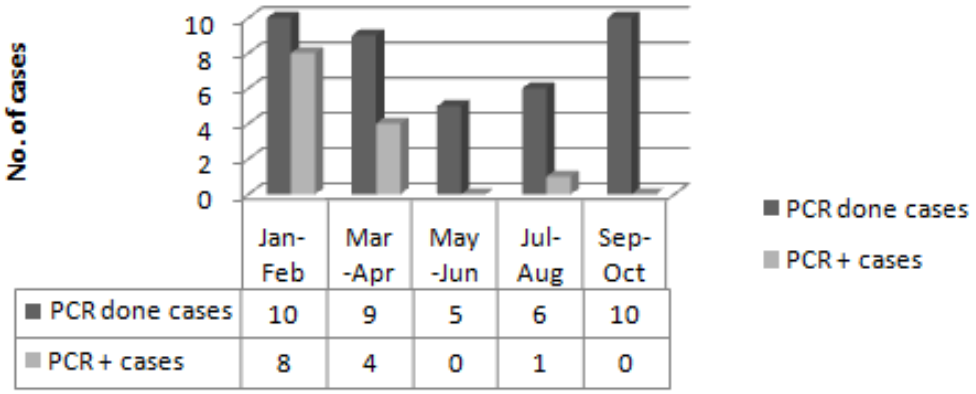
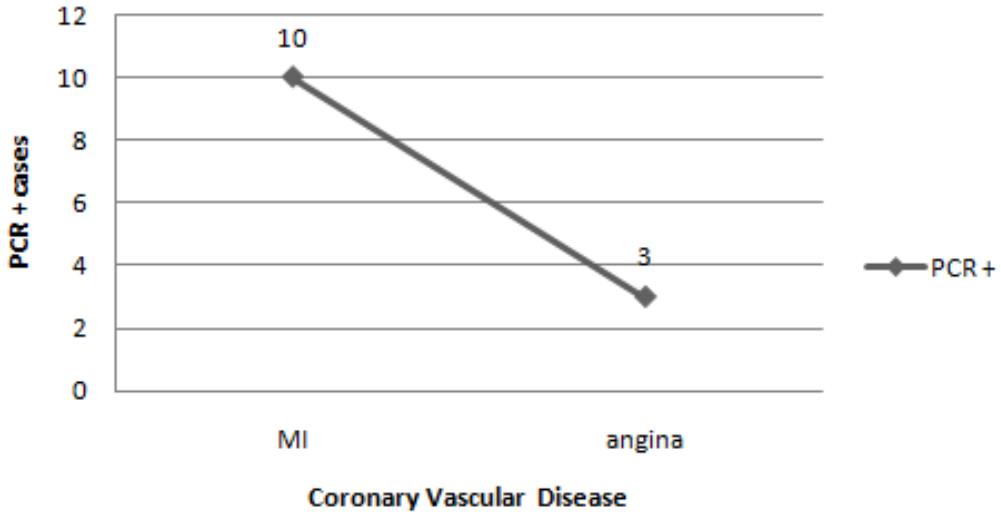


Figure 2. PCR positivity in patients. This diagram depicts PCR positivity in MI and angina patients. Of the PCR positive patients, 10 (76.9%) were MI and 3 (23%) were angina patients.



On analysing IFN-γ levels in CVD patients who were negative for *C. pneumoniae* infection by both serology and PCR, a higher level of IFN (19.41pg/ml) was observed in relation to healthy controls (11.32pg/ml). However, on comparing IFN-γ levels in patients with *C. pneumoniae* infection, the highest levels (55.21pg/ml) were observed where patients were seropositive for IgA as well as positive by PCR.

Discussion

In today's world where most deaths are attributed to non-communicable chronic diseases, more than half of these are as a result of coronary artery disease (CAD). Deaths from CAD are often premature and millions of non-fatal events result in disability. The known risk factors of coronary artery disease are not sufficient to explain all the epidemiological variables and fluctuations of the disease. These observations fuel renewed interest in a link between CAD and infectious agents.

Inflammation and immune activation are crucially involved in the pathogenesis of atherosclerosis and cardiovascular disease. We explored whether an infection with *C. pneumoniae* could be associated with an increase in IFN-γ levels thus inducing an inflammatory response.

Persistent *C. pneumoniae* infection was seen in 66.66% patients with CAD, which was statistically significant ($p < 0.05$). On analyzing IgA serology in CAD patients, the majority of patients with MI and angina were positive for persistent *C. pneumoniae* infection. Maximum seropositivity was seen in patients with MI (71.43%) followed by unstable angina (64.29%). Our results suggest that persistent infection may cause long-term vascular damage, leading to adverse clinical outcomes such as MI. The findings of this study suggest that *C. pneumoniae* seropositivity may be involved in the pathogenesis of cardiovascular disease, especially in cases of MI as maximum seropositivity was seen in patients of MI followed by angina.

In this study, 32.5% cases were positive for 16SrRNA. In comparison to an investigation by Wong *et al.*, we report a higher PCR positivity of 32.5% of which 76.92% were male and 23.07% were female. In our sample population traditional risk factors were not strongly associated with PCR positivity in patients of cardiovascular disease; however, the real picture may be different in a larger sample.

If we assume that PCR denotes active infection and IgA seropositivity demonstrates persistent infection respectively it appears that recurrence of *C. pneumoniae* infection is common. In our study there was a peak of infectivity as demonstrated by PCR in patients with MI (Figure 2.). We can hypothesize that in a subset of adults this recurrence of infection leads to excessive inflammatory response. Subsequent severe infection by *Chlamydia pneumoniae* may trigger widespread inflammatory response leading to myocardial infarction, explaining the second peak of PCR positivity.

Our study showed a seasonal variation with most of the PCR positive cases demonstrating acute infection in patients presenting in the winter months. This is also the time when maximum myocardial infarctions occur. Our study suggests that active *C. pneumoniae* infection occurs mostly in winter, predisposing the patient to excessive inflammatory atherogenic response which leads to adverse outcomes such as MI. PCR usually gives a good indication of the presence of intact viable pathogens as the DNA of dead bacteria is rapidly degraded by human restriction endonucleases. Although further research is required to confirm the high specificity obtained, the demonstration of circulating *C. pneumoniae* DNA in blood may be a useful prognostic test for a possible adverse outcome.

An interesting trend of increasing levels of IFN-γ with the severity and chronicity of cardiovascular disease is clearly visible in our study, as shown in Table 3. This may suggest an increasing role of inflammation and inflammatory markers not only in the initiation but also in the progression of cardiovascular diseases. On comparing IFN-γ levels in patients with *C. pneumoniae* infection, the highest levels (55.21pg/ml) were observed where patients were seropositive for IgA as well positive by PCR, thus emphasizing the key role of acute or persistent infection in elevating IFN-γ levels. IFN-γ levels were highest in patients with MI followed by angina patients, with the values increasing with severity of disease. On analyzing IFN-γ levels in CAD patients negative for *C. pneumoniae* infection, a higher level of IFN (19.41pg/ml) was observed in comparison to healthy controls (11.32pg/ml). Thus IFN-γ alone also appears to have a role in the pathogenesis of CVD.

Because *C. pneumoniae* is an intracellular pathogen, we felt that IFN-γ would be the first key cytokine to rise that could in turn activate the chain of events that could unleash a "cytokine storm" causing a worsening of atherogenesis. In chronic

Table 3. Detection of *C. pneumoniae* and inflammatory activity in cases and controls

Variable	Patients n (%)	Control n (%)	Chi-square *Kruskal Wallis	P-Value
C. pneumoniae IgA (Patients = 63 Control =40)				
Positive	42 (66.66%)	12 (30%)	13.18	< 0.001
Negative	21 (33.33%)	28 (70%)		
C. pneumoniae PCR (Patients = 40 Control =11)				
Positive	13 (32.5%)	0	4.79	< 0.05
Negative	27 (67.5%)	11(100%)		
IFN-γ levels (Patients = 63 Control =40)				
Mean IFN-γ levels (±SD) (pg/ml)	32.13 ± 51.2	11.32 ± 4.16	*5.78	0.33
Range (pg/ml)	8-638	7-17		
Mean IFN-γ levels based on diagnosis (±SD) (pg/ml)				
Stable angina	3.39	-	-	-
Unstable angina	12.91	-	-	
Myocardial infarction	23.89	-	-	

diseases such as in cardiovascular disease, biochemical reactions induced by Interferon-γ may have detrimental consequences for host cells [12]. Anti-inflammatory treatment (*e.g.*, with non-steroidal anti-inflammatory drugs or statins) may contribute to slowing down the adverse effects of IFN-γ [12].

However, the small sample size and the fact that PCR could not be performed in all patients in the present study considerably restrict our inferences. The role of other cytokines also needs to be studied.

This is a pilot study; therefore, a larger longitudinal study which focuses on *C. pneumoniae* infection with emphasis on acute exacerbations leading to inflammatory cytokine bursts would shed greater light on this issue.

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Corresponding author

Mehvash Haider
Dept of Microbiology, Jawaharlal Nehru Medical College, AMU Aligarh, India
Telephone: +918447560770, Fax: (0571)2400329
Email: mehvashaider@gmail.com

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