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EXPLORING THE RELATIONSHIP BETWEEN THE INFECTION OF C.*PNEUMONIAE* AND CORONARY ARTERY DISEASE

Ahed J Al-Khatib

Jordan University of Science and Technology, Faculty of Medicine, Irbid, Jordan

Majdi Al-Alawneh

Yarmouk University, Faculty of Science, Irbid, Jordan

Abstract

Atherosclerosis is considered an inflammatory disease, accordingly, there has been extensive research to determine whether microbial infections possibly contribute and maintain this inflammatory response. *Chlamydia pneumoniae* (*C. pneumoniae*), a common causative agent of respiratory tract infections, was associated with coronary artery disease (CAD) diseases. *C. pneumoniae* is a gram-negative bacterium which resembles viruses in terms of obligate intracellular growth. *C. pneumoniae* is able to infect several cell types, including circulatory monocytes, and by using monocytes as a vector, *C. pneumoniae* can disseminate from the lungs into extrapulmonary sites, including the subendothelium space of the arterial wall. In addition to inflammation lesion, development in the arterial wall requires macrophage transformation foam cells in the presence of an atherogenic cholesterol source like low-density lipoprotein (LDL) cholesterol.

The present investigation studied the relationship and prevalence of *C. pneumoniae* in patient with CAD who underwent to angiography. In this study, 130 patients were enrolled. Blood samples were taken from each patient, data collection, ELISA for IgG antibody and PCR for OMP1 gene have been carried out. The results showed that the prevalence of *C. pneumoniae* among the study sample was 56 out of 130 (43.1%) when ELISA is applied. However, prevalence of *C. Pneumoniae* decreased to 36 samples (27.7%) when the previous results were confirmed using PCR. This indicates that 65% of the ELISA positive samples were also PCR positive. The patients with CAD were considered as clinical symptomatic for *C. pneumoniae*, indicated that the organism used monocytes and macrophages as a vector, which lead to the acceleration of the atherosclerosis process. The total number of the patients, who

did not have any risk factor were 28 out of 130 patients (21.5 %). Twenty samples out of these 28 were positive in both ELISA and PCR (71.4 %),4 samples were positive in ELISA and negative in PCR(14.3 %), and 4 samples were negative in both ELISA and PCR (14.3%).

C. pneumoniae infection is considered as independent risk factor for CAD or atherosclerosis process. This study showed that there is an association between *C. pneumoniae* and CAD, between *C. pneumoniae* and diabetes, hypertension and smoking. On the other hand, it was clear that there is no association between *C. pneumonia*e and age, sex, living area, residence area, job, health food, rest pain, intermittent claudicating, obesity, hyperlipidemia, and environmental exposure.

Keywords: C. *pneumoniae,* Coronary Artery Disease (CAD), diabetes

Introduction

Chlamydia pneumoniae is a gram negative bacterium which resembles viruses in terms of obligate intracellular growth, which belongs to *Chlamidiaceae*. It is considered as a common cause of pneumonia. A third species of the genus *Chlamydia* causes acute respiratory disease and has been implicated on the basis of serological assay, as a cause of bronchitis, sinusitis and acute chest syndrome of sickle cell disease as well as asthma (Campbell *et al.,*1992). Furthermore, *C. pneumonia*e has been implicated as an inflammatory agent in atherosclerosis, a disease in which plaque builds up on the insides of arteries. Plaque is made up of fat, cholesterol, calcium, and other substances found in the blood. Over time, plaque hardens and narrows the arteries and the flow of oxygen-rich blood to organs and other parts of the body is reduced. This can lead to serious problems, including heart attack, stroke, or even death (Saikku, 2000; Kalayoglu, Libby and Byrne, 2002; Watson and Alp, 2008).

Development of foam cells from macrophages is a feature of atherogenesis. *In vitro* studies found that exposure of human macrophages to a combination of *C. pneumonia*e and LDL induced their transformation into foam cells (Wissler. 1995; Leinonen and Saikku. 1999). *C. pneumonia*e infection is ubiquitous and asymptomatic. Reinfection occurs commonly and antibodies against *C. pneumonia* are rare in children under the age of 5. Except in developing and tropical countries, approximately 50% of adults world wide have antibodies against *C. pneumoniae* and continues to increase slowly to 70% to 80% at ages 60 to 70 (Kuo, *et al.* 1995), and up to 10% of pneumonia patients were found infected with *C. pneumoniae* (Marrie, *et al* .1987; Grayston, *et al* .1986; Wang and Graystone, 1990). Patients

with coronary artery disease are significantly more likely to have serological evidence of past infection with *C. pneumoniae (*Saikku, *et al.* 1988; Saikku, et al 1992).

Diagnosis of *C. pneumoniae* infection is based on PCR assay, in which primers specific for *C. pneumoniae* are used. The nucleic acid sequence of the OMP1 of *C. pneumoniae* was determined (Moazed, *et al*. 1998).

The epidemiological infection of *C. pneumoniae* has been derived from serological studies using the *C. pneumoniae* specific microimmunofluorescence test (Kuo, *et al*. 1995). The expanding spectrum of *C. pneumoniae* infection has been related to clinical manifestation such as carotid artery stenosis, aortic aneurysm, cloudication, and stroke (Muhlestein, *et al*. 1999), and has been associated with other acute and chronic respiratory diseases as well as other clinical syndromes including erythema, nodosume, reiter syndrome, and sarcoidosis (Kuo, *et al*. 1995). These were determined by isolation or detection of the organism in specimen and seroepidimiological observation or combination of these methods (Molestina, *et al*. 1998). Immune complexes containing *C. pneumonia*e lipopolysaccharide have also been associated with 42 and 98 KDa *C. pneumonia*-specific antigens (Linnanm, *et al.* 1993); these associations have been extended to carotid artery disease and cerebrovascular disease (Danesh and Collins. 1997). *C. pneumoniae* antibodies have been associated with CAD and cerebrovascular disease, most were statistically significant, and the risk is independent of other CAD risk factors (hypercholesterolaemia, smoking, hypertension, diabetes, and family history).

Compelling evidence of the association of *C. pneumonia*e and atherosclerosis has been obtained by PCR, immunocytochemistry staining and electron microscopy, which have detected *C. pneumoniae* in atheromatuos lesion (Juvonen, *et al*. 1997).

Structures found within coronary atheromas were remarkably similar to the pear-shaped elementary body morphological characteristics described for *C. pneumoniae* (Grayston, *et al.* 1995).

The organism was found in tissues of male and female study participants of different ages and ethnic groups (Blasi, *et al.* 1996). This finding was confirmed by other investigations and also has found the organism in atherosclerotic lesion in iliac arteries and tissues from abdominal aortic aneurysms and aortic valve stenosis (Maass, *et al.* 1997; Jang, *et al*. 1993; Linnanm, *et al*.1993).

Other infectious agents including herpes simplex virus (HSV), cytomegalovirus (CMV), and *Helicobacter pylori* have been associated with cardiovascular disease. Only a

few studies have concurrently investigated the presence of *C. pneumonia*e and these infectious agents within lesion (Chiu, *et al*. 1997).

In a study investigating the presence of *C. pneumoniae*, CMV, and HSV in atherosclerosis and carotid artery, *C. pneumonia*e was also detected more frequently (71%) than CMV and HSV (35% and 10%, respectively) (Ong, *et al*. 1996). *C. pneumoniae* and CMV were independently associated with an increased risk for thrombosis (Chiu, *et al.* 1997).

Although *C. pneumonia*e and HSV or CMV may be found in the same lesion, *C. pneumoniae* has been more frequently found as the only infectious agent (Ong, *et al.* 1996).

The biological role of *C. pneumonia*e in atherogenesis was demonstrated by the presence of the organism. Three possibilities can be examined, first; the organism persist in vascular cells but does not contribute to pathologic abnormality, second; causes the initial injury and induces the atherosclerosis process, third; accelerates the severity or progression of the disease. If *C. pneumoniae* is involved, endothelial injury or activation resulting in monocyte/ macrophages adherence to the endothelium, migration to subendothelium, uptake of oxidized low-density lipoproteins transforming them into foam cells, and releases of cytokines have been occurred. These cytokines upregulate endothelial cell adhesion molecules leading to increased leukocyte adhesion, platelet aggregation at the site of endothelial damage resulting in the release of platelet-derived growth factor. This stimulates smooth muscle cell proliferation. Dedifferentiation of smooth muscle cells, which secrete collagen, elastin, and proteoglycans leads to the formation of fibrous tissue. The mature fibrolipid plaque consists of a lipid / cholesterol-rich core surrounded by fibrous cap composed of matrix elements (Grnhagen-Riska, *et al.* 1988).

Consistent with observations in animal models and humans, *in vitro* studies have demonstrated that vascular cells are susceptible to *C. pneumoniae* infection which produces productive infection in human macrophages, endothelial cells, and artery smooth muscle cells (Gaydos, 1996; Kalayoglu, 1998a).

In vitro studies have also investigated whether infection leads to the production of immunomodulators (Kalayoglu, 1998b). Another investigation obtained by *in vitro* study addressing a potential role of *C. pneumonia*e in atherogenesis found that exposure of human monocyte-derived macrophages to *C. pneumoniae* followed by addition of low-density lipoprotein resulted in foam cell formation, accumulation of cholesteryl esters, which accelerates the atherosclerosis process (Kalayoglu, 1998).

The ability of *C. pneumonia*e infection to induce the production of proinflammatory and procoagulate activities was investigated to determine its putative role in eliciting immune responses consistent with atherosclerotic processes (Kaukoranta, *et al.* 1996; Fong, *et al.* 1997).

Etiology can be established only through animal models or intervention studies. Rabbit and mice are susceptible to *C. pneumonia*e infection and provide well-defined model of atherosclerosis. Respiratory disease in both species is characterized by multifocal interstitial pneumonia. The disease is more severe and longer lasting in mice, and organisms are reisolated more readily from the lungs and aorta (Laitinen, *et al.* 1997). Early studies in rabbit and mice models indicate that *C. pneumoniae* infection can induce inflammatory changes similar to those of atherogenesis and augment the progression of the atherosclerotic lesion (Moazed, *et al.* 1998). There are many experiments demonstrated that DNA from dead organism is rapidly degraded, whereas live organisms survive within macrophages (Fryer, *et al.* 1997).

A causative role of *C. pneumoniae* infection in cardiovascular disease has not yet been firmly established, however, the high frequency of infection found in human atherosclerotic tissue in comparison to normal tissue, the induction and progression of atherosclerotic-like inflammatory changes in infected animal models of atherosclerosis, and early results from antichlamydial intervention studies in human are consistent with a causative role of *C. pneumoniae* in the disease process (Kim, *et al*. 2008).

*C. pneumonia*e was distinct from other two Chlamydial species that infect human, namely *C. trachomatis* and *C. psittaci*, this was clear by investigation of their diversity and immunoblot assay (Graystone, *et al*. 1975; Carter, *et al*. 1991**).**

Other studies indicated that there is an evidence for a relationship between coronary artery disease and *C. pneumonia*e .However, this issue needs more investigation (Danesh, and Collins, 1997).

Study objectives

1- To investigate the relationship between *C. Pneumoniae* and CAD patients in the Northern Jordan.

2- To study the prevalence of *C. pneumoniae* within the patient with CAD in the Northern Jordan.

Methods and subjects

Sample collection: venous blood was collected from patients of the cardiology clinic who underwent angiography in the king Abdullah University Hospital. Sex, age, and

diagnosis of each donor were recorded at the time of initial blood withdrawal. One hundred and thirty patients with cardiovascular disease were enrolled in this study. The control group was represented by 50 healthy donors.

As shown in (Table 1), there are 180 participants in this study among them 130 patients with CAD and 50 participants as control (no apparent health problems were noted or reported). Out of the CAD patients there were 48 female and 82 male participants. The mean age of the patients is 51 years. All patients are Jordanians. Living areas for patients included: city (57), villages (62) and desert (11) participants. The patients were coming from different cities in the north of Jordan among which are: Irbid 42 participants, Jerash 20, Ajloun 15, Mafraq 12 and others 41. Exercise training was performed by 78 patients while 52 patients did not. Regarding the control group, there were 10 females and 40 males. The mean age was 42 years. All control patients are Jordanians. Control participants live in: city (17), village (30) and desert (3) participants. Residence areas included: 36 participants in Irbid, 4 from Jerash, 2 from Ajloun and 8 from Mafraqe. Concerning the control group, 17 participants reported exercise training while 33 participants did not report exercise training.

Table 1:- Demographic characteristic of the study population:

Variables	*Frequency(No)*		*Percent (%)*	
	Patients (130)	**Control** (50)	**Patients**	**Control**
Sex: *Male* *Female*	**82** **48**	**40** **10**	**63** **37**	**80** **20**
Living area: *City* *Village* *Desert*	**57** **62** **11**	**17** **30** **3**	**43.8** **47.7** **8.5**	**34** **60** **6**
Residence area: *Irbid* *Jerash* *Ajloun* *Mafraqe* *Others*	**42** **20** **15** **12** **41**	**36** **4** **2** **8** **0.00**	**32.3** **15.4** **11.5** **9.2** **31.5**	**72** **8** **4** **16** **0.00**
Exercise activity: *Yes* *No*	**78** **52**	**17** **33**	**60** **40**	**34** **66**

PCR of blood mononuclear cells

EDTA blood was collected from the patients with coronary artery disease. Samples were stored overnight at 4ºC for the processing next day.

The plasma was clarified by centrifugation and the supernatant was stored at -27 ºC for serology. Blood cells were layered onto 3 ml of lymphoprep .The mononuclear cell layer, which also contained platelets was collected after centrifugation at 500 g for 15 min, washed twice in PBS and stored at -27 ºC. DNA was prepared according to Tong and Cillis (1993) from the blood mononuclear cells. The method implies addition of 8ml ice-cold cell lysis buffer to each 2 ml of blood. The mixture was then vortexed well and centrifuged for 10 min at 4000 rpm and 4ºC. The supernatant was separated carefully. This step was repeated. Eight ml of ice-cold pellet lysis buffer were added to each pellet, vortexed and centrifuged for 10 min. at 4000 rpm at 20 ºC. Then the supernatant was carefully taken. This step was also repeated again. Then 3 ml of nuclei lysis buffer, 120µl of proteinase K, 200µl of 10% SDS were added and the samples were incubated at 37ºC overnight. After the incubation period 1ml of the saturated NaCl solution was added and shaked vigorously for 20 sec and then centrifuged for 20 min. at 4000 rpm at 20 ºC. The supernatant was removed into a clean tube. Two volumes of ice-cold of absolute ethanol were then added to the supernatant and the vial was inverted several times. The DNA strands were removed into a clean 1.5 ml tube containing 500µl of 70 % ethanol, and centrifuged for 5 min at 7000 rpm at 20ºC. The pellet was taken after decanting the supernatant and was left to dry at room temperature and resuspended in 200 µl of DNA rehydration buffer for 2 hrs at room temperature and stored at -20ºC.

The following PCR programme was used; denaturation at 94ºC for 30 sec. primer annealing at 58ºC for 1.5 min, and extension at 72ºC for 1 min, followed by 45 cycles.

The following were added to the PCR tubes (treated sample and control sample):

- 12.5 µl master mix (master mix contains 20mM Tris HCl, pH 8.3, 1.5 mM magnesium chloride, 25mM potassium chloride, 50µM of each of the deoxynucleodise triphosphate (dATP, dCTP, dGTP, dTTP), 2 units Taq DNA polymerase)
- 1.3 µl OMP1 primer sense (Table 2).
- 1.3 µl OMP1 primer antisense (Table 2).
- 7.9 µl nuclease free water.
- 2 µl DNA sample.

PCR was performed using 3µl DNA solution. The amplification of OMP1 gene were carried out and the products were visualized by electrophoresis on 2% agarose gel electrophoresis, stained with ethidium bromide, and monitored using UV-transeluminator.

Table 2: The primer pair sequences that were used to amplify in this study:

Primer	**Sequence**	**Size of fragment (bp)**
omp1		**207**
Sense	**5'-TTA TTA ATT GAT GGT ACA ATA-3'**	
Antisense	**5'-ATC TAC GGC AGT AGT ATA GTT-3'**	

Enzyme –linked immunosorbent assay (ELISA)

C. pneumoniae-specific IgG antibody was detected by an rDNA lipopolysaccharide ELISA. This ELISA includes a chemically pure structure of a recombinant LPS, which contains a genus-specific epitope of the *C. Pneumoniae*. Microtiter plate wells are coated with *C. pneumoniae* antigens to bind corresponding antibodies of the specimens. After washing the wells to remove all unbound molecules, sample material (HRP) labeled anti-human IgG conjugate is added. This conjugate binds to the captured *C. pneumoniae*-specific antibodies. The immune complex formed by the bound conjugate is visualized by adding tetramethylbenzidine (TMB), substrate which gives a blue reaction product. The intensity of this product is proportional to the amount of *C. pneumonia*e-specific IgG antibodies in the specimen.

Sulphuric acid is added to stop the reaction. This produces a yellow end point color. Absorbance at 450nm is read using an ELISA microwell plate reader. (ELISA Fx10, made in USA)

Materials: kit (Nova Tec Immunodiagnostica GmbH)

Reagents

- *C. pneumoniae* coated wells (IgG): 12 breakpart 8-well snap-off strips coated with *C. pneumonia*e antigen.
- IgG sample diluents: buffer for sample dilution; PH 7.2±0.
- Stop solution: 0.2 mol/1 of Sulphuric acid.
- Washing solution: 20-fold concentrated buffer (PH 7.2±0.2) for washing the wells.
- *C. pneumoniae* anti-IgG conjugate: (HRB) labeled rabbit antibody to human IgG.
- *C. pneumonia*e IgG positive control
- *C. pneumonia*e IgG negative control.

- *C. pneumoniae* IgG cutt-off control.

Equipments

- ELISA microwell plate reader, equipped for the measurement of absorbance 450/620 nm (Kit from Nova Tec Immunodiagnostica GmbH).

Reagent preparation

- Solution with *C. pneumoniae* anti- IgG conjugate with horseradish peroxidase, buffer, stabilizers, preservatives and an inert red dye.
- Controls: the bottles labeled with positive, negative, and cut-off controls contain a ready to use control solution, each contains 0.1% Kathon.
- IgG sample diluents: contains phosphate buffer saline PH 7.3, stabilizers, preservative and an inert yellow dye, it is used for the dilution of the patient specimen.
- Washing solution: concentrated buffer, detergent, and preservatives.
- TMB substrate solution: contains tetramethylbenzidine/hydrogen peroxide system.
- Stop solution: contains 0.2 mM Sulphuric acid solution.
- Sample collection and preparation: serum samples were used with this assay.
- Sample dilution: all samples were diluted 1:100 with IgG sample diluents.

Assay procedure

- 100µl controls and diluent's sample was dispensed into their respective wells. A1 wells were left for substrate blank.
- The wells were covered with the foil, and incubated for 1 hr at 37ºC.
- After incubation, the foil was removed and the content of the wells was aspirated and each well was washed three times with 300µl of washing solution.
- 100µl of *C. pneumoniae* anti-IgG conjugate was dispensed into all wells except for the blank well and the wells were covered with foil and incubated for 30 min at room temperature.
- Washing of each well was repeated three times with 300µl of washing solution.
- 100µl of TMB substrate solution was dispensed into all wells.
- 100 µl of stop solution were dispensed into all wells.
- The absorbance of the specimen at 450/620 nm was measured within 30 min after addition of the stop solution.

Measurement

The ELISA microwell plate reader to zero was adjusted using the substrate blank well, then measured of all well at 450 nm; the absorbance value was recorded for each controls and patient sample.

Calculations

The cut-off is the mean absorbance value of the cut-off control determinations.

The cut-off results was (0.443/0.456) and the mean value of the cut- off control = 0.443+0.456÷2= 0.452

The samples were considered positive if the absorbance value equal or greater than the mean of absorbance value of the cut-off controls, and the samples were considered negative if the absorbance was below the cutt –off mean absorbance value.

Data analysis

SPSS program was used to explore association of the presence of *C. pneumoniae* DNA and conventional coronary risk factor with coronary artery disease of those subjects who does have coronary artery disease.

Results

CAD related factors

In this section of the study, there was a strong tendency to examine if the heart related factors under study are of important risk factors that might be associated with the pathogenesis of *C. pneumonia* or CAD. However, the heart related factors under study included the following factors as shown in Table 3: CAD symptoms, intermittent claudication, resting pain, heart problems, previous heart problems, brain problems, kidney problems, hypertension, diabetes, obesity, smoking, hyperlipidemia, environmental exposure and healthy food.

Table 3:- CAD related factor among the study and control group

Variable	**Frequency (no)**		**Percent (%)**	
	Patients (130)	**Control (50)**	**Patients**	**Control**
CAD symptoms				
Yes	55	0.00	42.3	0.00
No	75	50	57.7	100
Intermittent Caudication				
Yes				
No	50	4	38.5	8
	80	46	61.5	92
Rest pain				
Yes	62	0.00	47.7	0.00
No	68	50	52.3	100
Heart problems				
Yes	34	0.00	26.2	0.00
No	96	50	73.8	100
Brain Problems				
Yes	15	1	11.5	2
No	115	49	88.5	98
kidney Problems				
Yes	2	1	1.5	2
No	128	49	98.5	98
Hypertension				
Yes	66	7	50.8	14
No	64	43	49.2	86

Diabetes ***Yes*** ***No***	63 67	7 43	48.5 51.5	14 86
Obesity ***Yes*** ***No***	41 89	12 38	31.5 68.5	24 76
Smoking ***Yes*** ***No***	44 86	28 22	33.8 66.2	56 44
Hyperlipidemia ***Yes*** ***No***	50 80	15 35	38.5 61.5	30 70
Environmental Exposure ***Dust*** ***Chemical Materials*** ***Radiation*** ***Non of them***	37 1 0.00 92	13 1 0.00 36	28.5 0.8 0.00 70.7	26 0.8 0.00 72
Healthy Food ***Yes*** ***No***	78 52	35 15	60 40	70 30
Exercise activity ***Yes*** ***No***	78 52	17 33	60 40	34 66
Your home at ***City centre*** ***Village*** ***Desert*** ***Refugee camp***	57 62 11 0.00	17 30 3 0.00	43.8 47.7 8.5 0.00	34 60 6 0.00

ELISA results

130 sample patients with coronary artery disease were diagnosed using IgG *C. pneumonia*e antibody, out of 130 patients, 56 were seropositive for *C. pneumoniae* (43.1 %). Six healthy donors out of 50 (control group) were seropositive for *C. pneumoniae* (12%).

PCR results

Samples of CAD who were positive for *C. pneumoniae* using the ELISA were tested for OMP1 gene using PCR. Figure 1 and 2 illustrate the results of agarose gel electrophoresis for the amplified product of OMP1 gene for 36 patients out of 130. PCR positivity for *C. pneumoniae* was higher in CAD patients 27.6% compared with healthy control (0.00%) and all 36 positive PCR were highly positive in ELISA, indicating an active infection for *C pneumoniae*. Baseline clinical characteristics age, sex, gender, job, residence, asymptomatology, intermittent cloudication, rest pain, heart disease, brain disease, kidney

problem, hypertension, diabetes, obesity, smoking, hyperlipidemia, exposing to, healthy food and home site were compared in *C. pneumonia*e CAD patients and healthy control. No significant variation in positivity for *C. pneumonia*e were observed between age, sex, job, residence, asymptomatology, intermittent cloudication, rest pain, heart disease, brain disease, kidney problem, obesity, smoking, hyperlipidemia, exposing to, healthy food and home site. However, significant variations in positivity for *C. pneumoniae* between smoking, hypertension and diabetes were observed (tables 4-6).

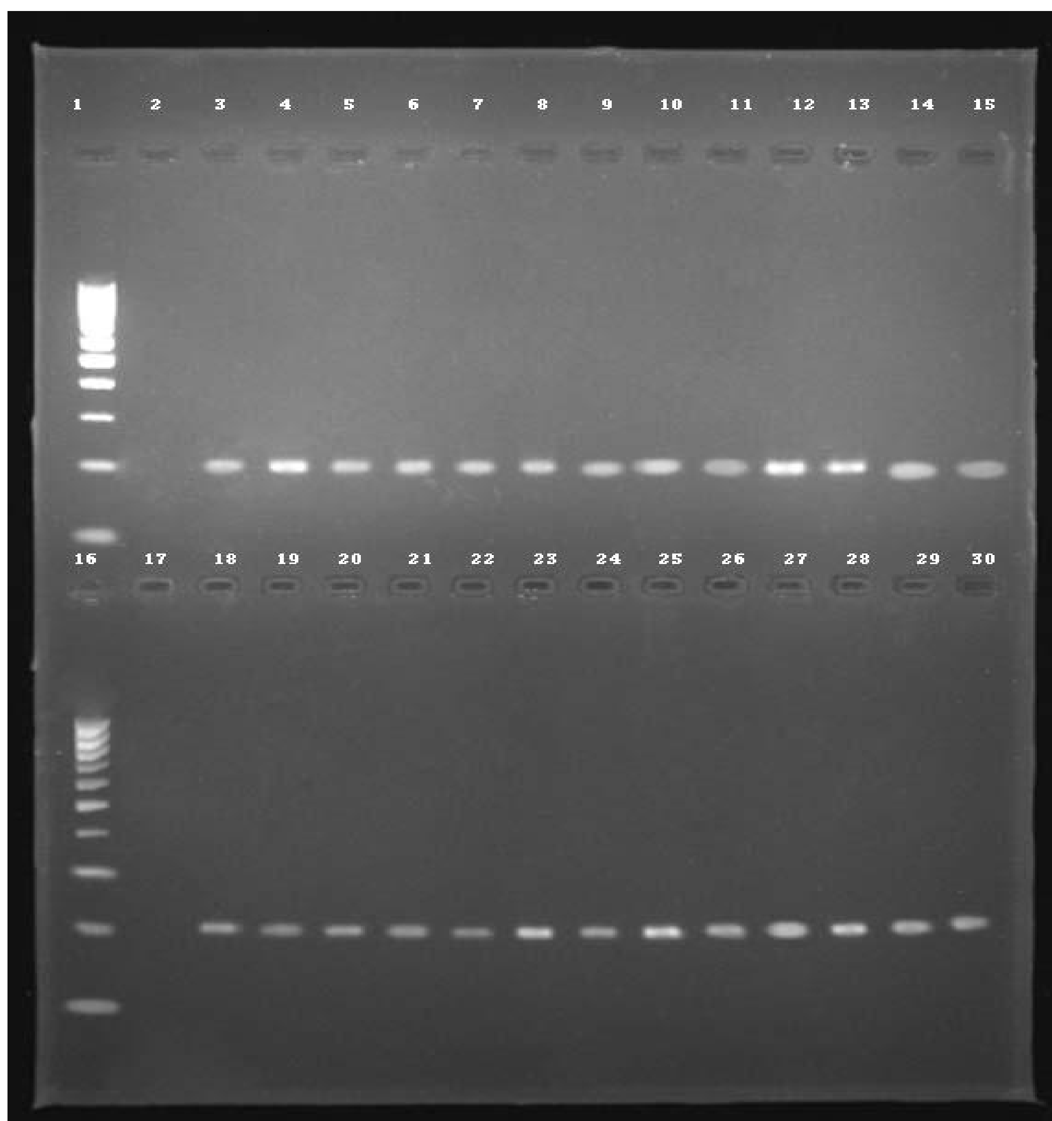

Figure 1*:* Agarose gel electrophoresis of PCR product of OMP1 gene (26 CAD samples).

Lane 1 and 16 represent 100 bp DNA ladder, lane 2 and17 represent negative control, lane(3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30) represent samples No(7, 8, 9, 11, 12, 13, 14, 17, 19, 21, 24, 25, 30, 34, 35, 37, 41, 58, 59, 66, 69, 71, 74, 75 and 83, respectively).

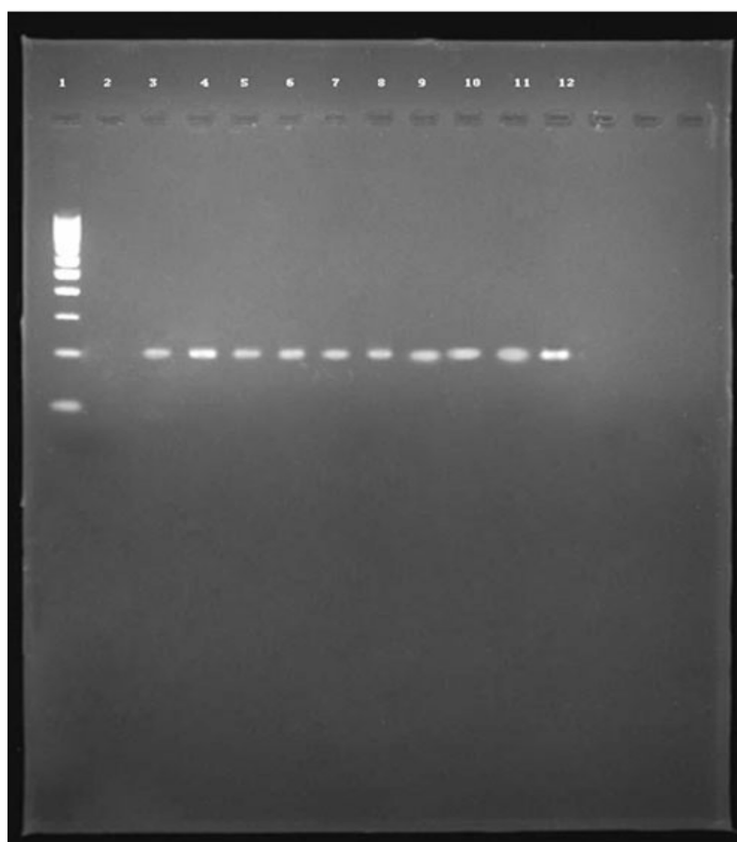

Figure 2: Agarose gel electrophoresis of PCR product of OMP1 gene (10 CAD samples): Lane 1 represent 100 bp DNA ladder, lane 2 represent negative control, lane (3, 4, 5, 6, 7, 8, 9,10, 11and 12) represents samples No (86, 91, 99, 100, 102, 103, 105, 110, 112 and 122, respectively).

Table 4:- Tests results of patients with CAD, with and without risk factors

Test results of patients with CAD.	Positive PCR	Negative PCR	Positive PCR with risk factor	Positive PCR without risk factor
Positive ELISA	36	20	20	16
Negative ELISA	0.00	74	0.00	0.00

Table 5:- negative relationship between CAD/*C. pneumoniae* and Study variables

Variables	P value*
Job and *C. Pneumoniae*	0.118
Residence area and *C. Pneumoniae*	0.104
Age and *C. Pneumoniae*	0.640
Gender and *C. Pneumoniae*	0.583
Environmental exposure and *C. Pneumoniae*	0.140
Healthy food and *C. Pneumoniae*	0.450
Living area and *C. Pneumoniae*	0.278
Asymptomatology and *C. Pneumoniae*	0.431
Intermittent claudication and *C. Pneumoniae*	0.317
Rest pain and *C. Pneumoniae*	0.119
Heart problem and *C. Pneumoniae*	0.657
Brain problem and *C. Pneumoniae*	0.721
Kidney problem and *C. Pneumoniae*	0.521
Obesity and *C. Pneumoniae*	0.657
Hyperlipidemia and *C. Pneumoniae*	0.159

* There is no significant association, P value > 0.05.

Table 6:- Positive relationship between CAD/*C. pneumoniae* and Variables

Variables	P value*
Diabetes and *C. pneumoniae*	0.032
Hypertension and *C. pneumoniae*	0.018
Smoking and *C. pneumoniae*	0.003
CAD and *C. pneumoniae*	0.001

* There is a significant association, P value < 0.05

Discussion

The role of microbes in inducing diseases has already been established. With time, other nonclassical roles of pathogens have been reported. For example, the relationship between cancers and some viruses as the role of human papilloma virus (HPV) in causing cervical carcinoma (Parkin. 2006). Another association between gastric ulcer and *Helicobacter Pylori* has also been reported (Cullen, *et al*. 1997). A large number of studies have been reported on the association between the obligate intracellular bacterium, *C. pneumoniae* and atherosclerosis (Saikku, et al. 1992).

In this study, other nonclassical role for *C. pneumoniae* with CAD was investigated to explore the relationship between *C. Pneumoniae* and CAD patients in the northern Jordan and the prevalence of *C. pneumoniae* among patients with CAD in the northern Jordan.

The results showed that the prevalence of *C. pneumonia*e among the study sample was 43.1% when ELISA was applied. The prevalence of *C. pneumonia*e decreased to 27.7% when the previous results were confirmed using PCR. Our findings are not consistent with other reported studies in literature. Hem, *et al* (2007) investigated the association between *C. pneumoniae* and CAD. The results of their study showed more positive results with PCR (29.6%) compared with the detection of circulating antibodies IgA and/or IgG (14%). George *et al* (2003) showed that out of 130 patients, only nine had viable *C. pneumoniae* in peripheral blood mononuclear cells (PBMCs) while 64 had serum IgG for *C. pneumoniae*. Although patients with atherosclerosis associated with *C. pneumoniae* had higher atherosclerotic scores, seropositive and negative patients showed similar scores. Patients with atherosclerosis exhibited higher inflammatory indices. Neither patients with detectable *C. pneumoniae* in PBMCs nor seropositive subjects had higher inflammation than negative patients**.** Melanie (2004) found that only two out of 43 (5%) patients with CAD had *C. pneumoniae* DNA presence within their peripheral blood mononuclear cell (PBMC) fraction using PCR analysis for OMP1 gene. Chlamydial antibodies were detected by ELISA**.** Also he showed that 95 % of the PCR positive patients exhibited positive IgG in ELISA test. In the present study 100 % of the PCR positive patients for *C. pneumoniae* OMP1 gene, were also IgG positive in ELISA test.

Our PCR results showed that 36 samples were positive for OMP1 specific *C. pneumoniae* (65%). These samples were also positive in ELISA test.

The samples, which gave the IgG titer below 0.600 O.D in ELISA reading, did not give positive PCR 0.00%, while the samples, which showed negative PCR and positive ELISA may be considered as samples from patients with sub-clinical symptoms for *C. pneumoniae* according to O.D value (low positive). On the other hand, samples that were positive in both ELISA and PCR (above 0.613) were considered as samples from patient with chronic *C. pneumoniae* infection.

All negative ELISA samples for IgG antibodies for *C. pneumoniae* were also PCR negative. This result proved that when *C. pneumoniae* was able to use macrophages and monocytes as dissemination vectors, the atherosclerosis process is accelerated.

The total number of patients, who did not have any risk factor, were 28 out of 130 patients (21.5 %). Twenty samples out of 28 were positive in both ELISA and PCR (71.4 %). Four samples from 28 samples were positive in ELISA and negative in PCR (14.3 %). Four samples from 28 samples were negative in both ELISA and PCR (14.3%).

The Control group which was enrolled in this study consisted of 50 healthy donors, 10 females and 40males. The mean age was 42 years, and they were subjected to the same protocol applied to the patients.

In ELISA test, 56 samples of patients gave positive results for IgG antibodies and 6 samples of the control group gave positive results in ELISA and the IgG titers were below 0.600 O.D. This result was confirmed by PCR (amplification for OMP1 gene), in which all samples were PCR negative. The 6 samples out of 50 control group, which were positive in ELISA and negative PCR may be from patients who are sub-clinical symptomatic for *C. pneumoniae* infection.

In our study, 36 sample out of 56 (65%) were ELISA positive and PCR positive, that indicates an active infection or clinical symptoms for *C. pneumoniae* infection, which may points to a participation in the atherosclerosis process. The results may reflect that clinical symptoms of infection appear when *C. Pneumoniae* is able to use the monocytes and macrophages as a vector for its dissemination.

According to the results of the present investigation *C. pneumoniae* infection can be considered as independent risk factor for CAD or atherosclerosis process. There is an association between *C. pneumoniae* and CAD, diabetes, smoking and hypertension. On the other hand, we found that there is no association between *C. pneumonia* and age, sex, living

area, residence area, job, health food, rest pain, intermittent cloudication, obesity, hyperlipidemia, and environmental exposure.

Conclusions

1- Both PCR and ELISA tests for *C. pneumoniae* indicated that there is a significant correlation with CAD.

2- According to our results and their statistical analysis *C. pneumoniae* is considered as an independent risk factor for CAD and atherosclerosis.

3- Presence of *C. pneumoniae* DNA in peripheral blood mononuclear cells, means that this bacteria used the Mononuclear cells as a vector for the dissemination when the infection reaches at high level.

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