

Original Article

Lack of strong association of *Chlamydia pneumoniae* and atherosclerosis in a Jordanian population

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Abstract

Introduction: The correlation of *Chlamydia pneumoniae* to coronary artery disease (CAD) in Jordan was investigated in this study.

Methodology: Totals of 361 atherosclerotic patients and 392 apparently healthy controls of both sexes were enrolled. *C. pneumoniae*-specific IgG antibodies were measured by the microimmunofluorescence assay (MIF). The presence of the bacterial DNA in the blood by polymerase chain reaction (PCR) was also tested.

Results: The overall IgG seroprevalence, estimated at a titer of 1/16, was insignificantly higher in patients (75.9%) than in controls (71.7%). About 59.3% of patients demonstrated seropositivity at titers $\leq 1/256$, which are suggestive of chronic or presumed past infection, whereas 54.1% of controls were seropositive at these titers ($p > 0.05$). Analysis of gender-specific seroprevalences revealed no obvious relation between *C. pneumoniae* and atherosclerosis in males (78.9% and 77.9% in atherosclerotic and control males, respectively; $p > 0.05$). However, a significantly elevated seropositivity was detected in atherosclerotic females (71.7%) compared with control females (64.2%). On the other hand, the PCR-based detection of *C. pneumoniae* DNA failed to correlate the bacterium to atherosclerosis.

Conclusions: We were unable to show a strong association between *C. pneumoniae* and CAD, potentially because of the presence of high seroprevalence of *C. pneumoniae* antibodies and the unreliability of the whole blood-based nested PCR technique used.

Key words: atherosclerosis; *Chlamydia pneumoniae*; prevalence; seropositivity; polymerase chain reaction.

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Introduction

Atherosclerosis is one of the most important leading causes of death, especially in developed countries [1]. It is now widely accepted that inflammation plays a role in the pathogenesis of atherosclerosis [2,3]. Therefore, numerous reports have recently focused on consequences of infection of a wide variety of viruses and bacteria in the genesis and progression of atherosclerosis [4,5]. Among these pathogens is *C. pneumoniae*, an obligate intracellular bacterium, which is responsible primarily for pulmonary infections such as sinusitis, pharyngitis, and pneumonia [6,7].

Based on the detection of elevated levels of specific anti-*C. pneumoniae* IgG in sera collected from patients with coronary heart disease (CAD), a study published in 1988 first proposed an association between *C. pneumoniae* and atherosclerosis [8]. Subsequently,

considerable seroepidemiological studies have been performed to implicate this pathogen in heart vascular disease [9,10]. In addition, different lines of evidence on the existence of this link have been provided by demonstration of *C. pneumoniae* nucleic acids and the bacterium in atherosclerotic plaques by polymerase chain reaction (PCR), electron microscopy, immunohistochemistry, and culturing of the bacterium from the atheroma [4,11-14]. Moreover, some *in vivo* studies have demonstrated that *C. pneumoniae* infection may accelerate the progression of atherosclerotic plaques in animal models [4,14].

Many *in vitro* studies tried to support the role of *C. pneumoniae* in cardiovascular disease and in endothelial dysfunction. This pathogen was found to replicate within vascular cells, such as macrophages, smooth muscle cells, and endothelial cells [12,14]. *C.*

pneumoniae was also found to elicit inflammatory cytokines, inducing chronic inflammation [15]. Furthermore, *C. pneumoniae* has been shown to induce foam cell formation, smooth muscle cell multiplication, platelet aggregation, and production of reactive oxygen intermediates and cellular adhesion molecules [15,16]. Paradoxically, other *in vivo* and *in vitro* studies have failed to link *C. pneumoniae* with atherosclerosis [17] and, therefore, it remains controversial whether *C. pneumoniae* could actively play a role in vascular disease development or is an innocent bystander.

Epidemiological studies carried out worldwide demonstrated detection rates of anti-*C. pneumoniae* IgG in apparently healthy individuals, which ranged from 40% to 86%, indicating that *C. pneumoniae* infection is ubiquitous [18,19]. *C. pneumoniae* is characterized by its ability to systemically disseminate from the primary site of infection (the lung) through the circulatory system, and to localize in nonpulmonary tissues such as vascular walls [20,21]. For this dissemination, the pathogen most likely utilizes peripheral blood monocyte cells (PBMCs) as a vehicle [20,21].

The majority of reports that provided evidence on the role of *C. pneumoniae* in CAD used the microimmunofluorescence (MIF) test, which is considered by the Centers for Disease Control and Infection in USA and Canada to be the only currently acceptable serological method [14,22]. The second approach widely used to confirm this association is the detection of the pathogen's nucleic acid in the bloodstream by PCR [13,23,24].

The present study was designed to investigate whether *C. pneumoniae* was a risk factor for atherosclerosis in Jordanian patients who suffered from coronary heart disease, based on the differences in the detection rates of anti-*C. pneumoniae* IgG and *C. pneumoniae* DNA in the whole blood between patients and control subjects.

Methodology

Patients and controls

A total of 361 patients recruited from cardiac surgery and cardiology clinics, the University of Jordan Hospital, Amman, Jordan, were enrolled in this study (mean age 61.0 years). All patients had symptomatic coronary artery atherosclerotic disease and underwent either coronary artery bypass grafting (CABG) or coronary artery stenting. The atherosclerotic patients included 209 males (mean age 60.0 years) and 152 females (mean age 62.4 years). A total of 392 age- and sex-matched non-atherosclerotic controls, who visited

the outpatient clinics at the hospital during December 2008 and May 2009 for various reasons, were also included (mean age 59.5 years). Apparently healthy control subjects comprised 213 males (mean age 59.6 years) and 179 females (mean age 59.3 years). Individuals who had symptoms of respiratory or cardiovascular disease or had received antibiotics within the last three months preceding enrollment were not included as controls. Based on their ages, patients were divided into four age groups: 40–49 years ($n = 49$), 50–59 years ($n = 99$), 60–69 years ($n = 140$), and ≥ 70 years ($n = 73$). Control subjects were also distributed in similar age brackets: 40–49 years ($n = 79$), 50–59 years ($n = 106$), 60–69 years ($n = 132$), and ≥ 70 years ($n = 75$). Informed consent was obtained from all study participants. This study was approved by the ethics committee at the University of Jordan Hospital.

Serum collection

Whole blood samples were drawn from study population in EDTA-treated gel-containing tubes (Greiner Bio-One, Stonehouse, UK). Serum specimens were separated by centrifuging blood samples at 3,000 rpm; these samples were then stored at -20°C until analyzed.

DNA extraction from whole blood

DNA from a second whole blood sample collected from all study subjects was isolated using i-genomic Blood DNA Extraction Mini Kit (iNTRON Biotechnology, Gyeonggi-Do, Korea) according to the manufacturer's instructions. DNA was eluted in 50 μL , quantified on a spectrophotometer (Biotech Engineering Management, Nicosia, Cyprus), and stored at -20°C until tested.

PCR

The presence of *C. pneumoniae* DNA was examined using a nested PCR method recommended by Fukano [25]. This method was based on targeting the gene encoding 53 kDa outer membrane protein. The outer primers were 5' ATG ATC GCG GTT TCT GTT GCC A 3' (forward) and 5' GAG CGA CGT TTT GTT GCA TCT C 3' (reverse). The internal primers were 5' TGT CCA AGC GGT GAA ACA AG 3' (forward) and 5' CAA CCG TGA CCC ATT TAC TG 3' (reverse). Amplicon sizes were 499 bp and 239 bp for the first and second rounds of amplification, respectively. Each amplification reaction was performed in a volume of 40 μL , containing 500 ng of extracted DNA and 1 μL of each primer, using a LifePro thermocycler (Bioer, Binjiang, China). For the first (outer) amplification,

cycling consisted of 30 seconds at 95°C followed by 40 cycles of 1 minute at 95°C, 1 minute at 56°C, and 1 minute at 72°C. The inner amplification consisted of 30 seconds at 95°C followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 56°C, and 1 minute at 72°C. All amplification products were analyzed by agarose gel electrophoresis, and DNA bands were then visualized in a gel documentation system (Clever Scientific, Warwickshire, UK).

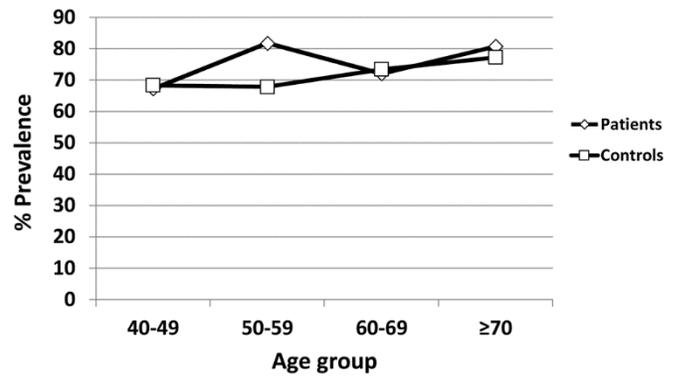
Antigen preparation

The *C. pneumoniae* strain VR1310, generously provided by Thomas F. Meyer, Max Planck Institute for Infection Biology, Berlin, Germany, was used for antigen preparation in the serological testing method MIF. Antigens were whole elementary bodies prepared as described previously [26], with minor modifications. Briefly, *C. pneumoniae* were grown in cell culture, purified by centrifugation, and treated with 0.05% formalin. Before dotting onto wells of immunofluorescence slides, antigens were mixed in 0.5% yolk sac. The dotted slides were dried and fixed with acetone for 15 minutes at room temperature and then stored at -70°C until used.

Serological assay

The MIF test is based on the indirect detection of human IgG antibody against *C. pneumoniae* using labeled anti-human IgG [26]. IgG antibody in sera was first screened at 1:16, a dilution considered to be a marker for *C. pneumoniae* positivity in this study.

Figure 1. Seroprevalence of IgG antibodies against *C. pneumoniae* in Jordanian control subjects and atherosclerotic patients of both sexes according to age.



Positive and negative control sera obtained previously from Jordanian individuals [19] were also applied in each run. Slides were incubated in a humid chamber at 37°C for one hour. After washing with phosphate-buffered saline (PBS) to remove unbound serum antibodies, each antigen spot was overlaid with fluorescein-labeled goat anti-human IgG antibody (Bio-Rad, Hercules, USA) and incubated as before. Slides were then washed, dried, mounted, and examined using an epifluorescence microscope (Nikon, Tokyo, Japan) at 400x magnification. If sera were reactive at 1:16, they were further tested at serial twofold dilutions (from 1:16 to 1:512) for IgG antibody titer determination. Chlamydial IgG antibody titers of 1:16 to 1:256 were considered indicative of chronic or presumed past infection, and a titer of 1:512 was defined as a marker for recent or possible acute infection [7,19,22,27,28].

Table 1. Prevalence of anti-*C. pneumoniae* IgG antibodies in healthy controls by age and gender.

Age group (years)	Males			Females			Both genders			Males versus females (p)*
	No. examined	No. positive ^a	% positivity	No. examined	No. positive ^a	% positivity	No. examined	No. positive ^a	% positivity	
40-49	42	33	78.6	37	21	56.8	79	54	68.4	0.761
50-59	57	38	66.7	49	34	69.4	106	72	67.9	0.277
60-69	71	58	81.7	61	39	63.9	132	97	73.5	0.885
≥ 70	43	37	86.0	32	21	65.6	75	58	77.3	0.465
Total	213	166	77.9	179	115	64.2	392	281	71.7	0.176

^aIgG ≥ 1:16; *The difference in seropositivities between males and females within the same age group was statistically significant when p <0.05.

Table 2. Prevalence of anti-*C. pneumoniae* IgG antibodies in atherosclerotic patients by age and gender

Age group (years)	Males			Females			Both genders			Males versus females (p)*
	No. examined	No. positive ^a	% positivity	No. examined	No. positive ^a	% positivity	No. examined	No. positive ^a	% positivity	
40-49	36	25	69.4	13	8	61.5	49	33	67.3	0.068
50-59	57	50	87.7	42	31	73.8	99	81	81.8	0.781
60-69	76	56	73.7	64	45	70.3	140	101	72.1	0.327
≥ 70	40	34	85.0	33	25	75.8	73	59	80.8	0.684
Total	209	165	78.9	152	109	71.7	361	274	75.9	0.033

^aIgG ≥ 1:16; *The difference in seropositivities between males and females within the same age group was statistically significant when p <0.05.

Statistical analysis

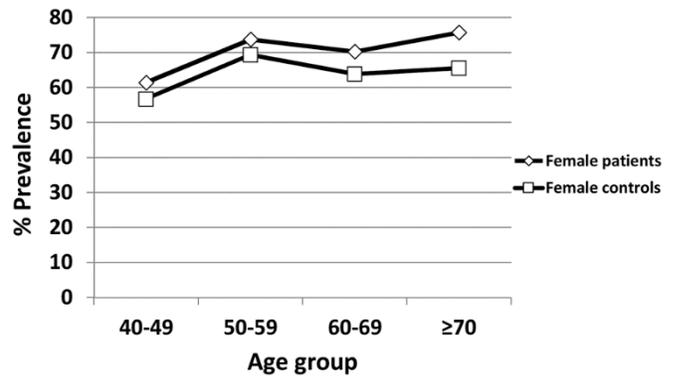
The differences in the IgG seropositivities obtained in control individuals and atherosclerotic patients were statistically analyzed using the Chi-square test. A p value < 0.05 was considered statistically significant.

Results

The relationship between C. pneumoniae serology and atherosclerosis

The link between *C. pneumoniae* and atherosclerosis was first examined based on the seroprevalence of IgG in Jordanian patients with confirmed CAD. The seropositivity of *C. pneumoniae* antibodies in sera from a total of 361 patients was compared with that in sera from 392 controls. Tables 1 and 2 summarize the presence of *C. pneumoniae* IgG antibody in control subjects and atherosclerotic patients, based on age and gender. The statistical differences, if present, in IgG seropositivity between males and females within the same age group were determined and indicated in Tables 1 and 2. Both genders within each age group of controls or patients were combined, and IgG detection rates were also demonstrated (Tables 1 and 2 and Figure 1). By comparing seropositivities (indicated by IgG titers $\geq 1/16$) between patients and controls of both sexes, almost comparable IgG detection rates were found in the age groups 40–49 years (67.3% versus 68.4%; $p < 0.05$) and 60–69 years (72.1% versus 73.5%; $p > 0.05$). However, the IgG prevalence in the patients 50–59 years or ≥ 70 years of age exceeded 80%, a percentage that was insignificantly higher ($p > 0.05$) than that obtained for the respective control groups (Tables 1 and 2). Moreover, IgG antibodies determined at $\geq 1/16$ titers were detected in 75.9% of atherosclerotic patients, compared with 71.7% of control subjects (Tables 1 and 2). This difference was statistically insignificant ($p > 0.05$). Taken together, though higher detection rates of IgG in patients with confirmed atherosclerosis than in controls were observed, statistical analysis points to the

Figure 2. Comparison between seroprevalence of anti-*C. pneumoniae* IgG antibodies in Jordanian control and atherosclerotic females.



existence of a weak association of *C. pneumoniae* with atherosclerosis in the general Jordanian population.

According to previously published reports on MIF-based estimation of *C. pneumoniae* seroprevalence, IgG titers from 1/16 to 1/256 are considered indicative of chronic or presumed past infection. IgG titers of $\geq 1/512$ suggest evidence for recent or possible acute infection with *C. pneumoniae*. Table 3 illustrates the distribution of endpoint titers of *C. pneumoniae* IgG among atherosclerotic and control subjects. Extrapolated from the data shown in the table, 59.3% of atherosclerotic patients showed positivity at titers $\leq 1/256$, indicating chronic or presumed past infection, whereas 54.1% of controls were seropositive at the indicated titers ($p > 0.05$). In contrast, the percentage of IgG positive cases detected at 1/512, suggestive of recent or possible acute infection, was insignificantly higher in the control group than in patients (17.6% versus 16.6%; $p > 0.05$). Overall, the rate of prevalence of IgG titers indicative of chronic or presumed past infection was found to be slightly higher, but insignificant, in the patient group than in the control group. This finding does not strongly support a possible role of chronic infection or past exposure to *C. pneumoniae* in atherosclerosis in the whole Jordanian population.

Table 3. Distribution of seropositive IgG titers against *C. pneumoniae* in atherosclerotic patients and control individuals^a

IgG titer	Atherosclerotic patients (n = 361)		Control subjects (n = 392)	
	No.	Percentage	No.	Percentage
1/16	12	3.3	25	6.4
1/32	42	11.6	36	9.2
1/64	49	13.6	47	12
1/128	54	15	48	12.2
1/256	57	15.8	56	14.3
1/512	60	16.6	69	17.6

^aDifference between percentages of atherosclerotic patients seropositive at IgG titers from 1/16 to 1/256, indicative of chronic or presumed past infection, compared to that in controls is not significant ($p < 0.05$).

Further, the results of seropositivities obtained for each gender in both groups of study subjects were compared. As depicted in Tables 1 and 2, a negligible higher overall prevalence was noticed in atherosclerotic males compared with control males (78.9% versus 77.9%; $p > 0.05$). However, careful analysis of prevalence findings obtained for control and atherosclerotic males revealed that IgG detection rates were higher in the control males in all age brackets compared with male patients of corresponding ages ($p > 0.05$), except the age group 50–59 years ($p > 0.05$). Paradoxically, significant elevated overall seropositivity was detected in atherosclerotic females compared with control females (71.7% versus 64.2%, respectively; $p < 0.05$). Evidently, the IgG detection rates were considerably higher in atherosclerotic females in all age groups, when compared with those in control females (Figure 2). Taken together, the present work provides no evidence for an association between *C. pneumoniae* and atherosclerosis in males based on IgG prevalence. Intriguingly, however, the anomalous findings obtained for females may indicate a possible contribution of the pathogen in atherosclerosis, at least in females. These data point to a potential gender-specific role of *C. pneumoniae* in atherosclerosis.

Of note, a clear relationship between gender and the seroprevalence of the *C. pneumoniae* IgG antibody was found in both the control and patient groups. In the control group, the overall prevalence in males was noticeably higher than in females (77.9% versus 64.2%, respectively; $p > 0.05$), as indicated in Table 1. This elevated detection rate in males was observed in all age groups, except the 50–59 years group (Table 1). This association between gender and IgG seropositivity was also confirmed in the patient group (Table 2), in which higher prevalence rates among males were observed in all age groups, with an overall prevalence of 78.9% compared with 71.7% in females ($p < 0.05$). Statistical differences between both genders in each age group are shown Tables 1 and 2.

Chlamydial DNA detection in the whole blood of control and atherosclerotic individuals

The possible use of *C. pneumoniae* DNA detection in whole blood samples as a potential marker of atherosclerosis was tested. Demonstration rates of bacterial DNA in the different age groups, which served as controls, ranged from 6.3% to 13.2%, whereas DNA positivity in blood from atherosclerotic age groups ranged from 4.1% to 10.7%. Overall, 28 patients (7.8%) were *C. pneumoniae* DNA positive compared with 36 control subjects (9.2%). Of those PCR-positive controls

and patients, only 2 control individuals were serologically *C. pneumoniae* negative. No agreement was found between detection of chlamydial DNA in the whole blood and atherosclerosis, suggesting that PCR of whole blood is not a good tool to link *C. pneumoniae* with atherosclerosis.

Discussion

The present study addressed the possible link between *C. pneumoniae* and CAD in Jordanian patients of both genders based on the assessment of serum IgG levels and the detection of bacterial nucleic acids in the whole blood. Determined at a cut-off value of 1/16, slightly higher insignificant *C. pneumoniae* IgG prevalence was observed in CAD patients than in controls. In addition, PCR results could not associate *C. pneumoniae* with CAD.

The first study associating *C. pneumoniae* infection and atherosclerosis was performed in 1988 [8]. In that study, patients with chronic stable CAD or acute myocardial infarction were significantly more likely to have elevated *C. pneumoniae* IgG prevalence (68%) than were controls (17%). This was followed by many cross-sectional retrospective serological studies that suggested a role for chronic *C. pneumoniae* infection in the initiation and progression of CAD in different populations worldwide [10,29]. Importantly, however, some seroepidemiological reports yielded controversial results. Meta-analyses of seroepidemiological studies published in 2000 and 2002 indicated a weak or no relationship between elevated anti-*C. pneumoniae* antibody titers and cardiovascular events [30,31]. Similarly, based on the detection of IgG at titers $\geq 1/16$, we found a poor association between IgG seropositivity and CAD in this case-controlled population-based investigation.

Previous epidemiological studies indicated that *C. pneumoniae* antibody prevalence is 50% by the age of 20 and increases with increasing age [18]. In Jordan, a population-based investigation indicated an overall prevalence of *C. pneumoniae* of 54.9% [19]. Undoubtedly, the seroprevalence of infection in Jordan is relatively low in children and steadily increases to reach more than 65% in healthy middle-aged and elderly persons [19]. The current study was performed in relatively elderly population and showed a slightly higher insignificant prevalence of *C. pneumoniae* IgG in CAD patients than in controls (75.9% versus 71.7%). Because of the apparent high overall *C. pneumoniae* IgG seropositivity in Jordan, correlating *C. pneumoniae* antibody levels with CAD, based on the cut-off value of 1/16, seems to be generally problematic and

complicates the interpretation of our results. Nevertheless, serological findings obtained in this study most likely do not necessarily suggest lack of involvement of *C. pneumoniae* in atherosclerosis. Therefore, other approaches, such as direct detection of the pathogen in atheromatous plaques by culturing and non-culture techniques, should alternatively be utilized in Jordan and in areas where *C. pneumoniae* infection in the general population is extremely common to analyze the possible role of *C. pneumoniae* in atherogenesis.

Previous seroepidemiological reports investigating asymptomatic subjects indicate that *C. pneumoniae* antibody seropositivity rates tend to be higher in men than in women [7,19]. Here, the same observation was obtained in the control and patient groups, where men had a higher frequency of IgG, indicating that men are more susceptible to *C. pneumoniae* infection than women. Interestingly, men were found to also have a higher prevalence of cardiovascular disease than women [32]. In the present study, we analyzed the detection rates of *C. pneumoniae* IgG in each gender separately. As shown in Tables 1 and 2, serological findings obtained for males in patient and control groups were almost comparable (78.9% versus 77.9%), indicating no relationship between *C. pneumoniae* and CAD in this gender. Intriguingly, a significant prevalence rate of IgG was detected in CAD females compared with control females (71.7% versus 64.2%), suggesting a potential role of *C. pneumoniae* in atherosclerosis in females but not males. In this context, another gender-specific difference was published by Sakurai-Komada *et al.* [33], who correlated high serum *C. pneumoniae* IgA titers with a higher risk of coronary heart disease mortality in females. Taken together, our results along with those reported by Sakurai-Kumada *et al.* [33] may suggest gender-specific variations in the role of *C. pneumoniae* infection in the establishment or in promoting the clinical consequences of atherosclerosis. Further investigations are required to confirm these preliminary findings on possible gender-specific variations and to, subsequently, provide an explanation for them.

Besides seroepidemiology, the contribution of *C. pneumoniae* to cardiovascular disease has also been debated in a range of studies, including histopathology, animal models, and clinical intervention trials [10,17]. For instance, while some studies pointed to little or no evidence for the presence of *C. pneumoniae* in atheromas, the majority of histopathological studies concluded that *C. pneumoniae* could be detected in atherosclerotic plaques, unlike in healthy arteries

[10,34-36]. To date, it is not clear whether differences between these studies are attributable to variations in the populations examined or in the tools used to determine the role of the pathogen in cardiovascular disease.

Several studies propose that *C. pneumoniae* may represent a risk factor for atherosclerosis or its complications based on the detection of the pathogen in the circulatory system. *C. pneumoniae* presence was confirmed in PBMCs, including monocytes and lymphocytes, by a PCR technique [20,37]. The pathogen presence in PBMCs may indicate a current infection or it may be harbored within these cells in a persistent state [37-39]. More than 20 studies have investigated the prevalence of *C. pneumoniae* DNA in PBMCs in cardiovascular patients and controls from different populations. In these studies, the prevalence ranged from 4%–87% in patients and from 0%–50% in controls [10,14]. In addition to the data published above on PBMCs testing, Petyaev *et al.* successfully confirmed the presence of *C. pneumoniae* by culturing methods and PCR in serum samples of patients with acute coronary syndromes [40]. Here, we tried to demonstrate *C. pneumoniae* in the bloodstream by isolating the bacterial DNA from the whole blood. The nested PCR assay used here detected *C. pneumoniae* DNA in only 7.8% of CAD patients and 9.2% of controls. This study clearly revealed that PCR using whole blood samples is less efficient than serology in the identification of *C. pneumoniae* carriers and, accordingly, is not a reliable tool to link this pathogen with CAD.

Conclusions

Because of the noticeable high seroprevalence of *C. pneumoniae* infection in the middle-aged and elderly nationals enrolled, the present study could not provide strong evidence for the association between *C. pneumoniae* and CAD in Jordan. Besides, detection of *C. pneumoniae* in the whole blood seems to be neither a practical measure of infection nor a tool to determine the role of *C. pneumoniae* in CAD. Future investigations, including more useful tools than serology and whole blood-based PCR, are suggested to find a role for the pathogen in the establishment of atherosclerosis or in promoting the clinical consequences of this disease in Jordan. Furthermore, the possible involvement of other infectious agents and risk factors in CAD in Jordanian individuals should also be examined.

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