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Investigation of *Chlamydia pneumoniae* infection in Moroccan patients suffering from cardiovascular diseases

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ABSTRACT

Chlamydia pneumoniae is an intracellular bacterium responsible for respiratory diseases and is highly involved in cardiovascular disease development, mainly atherosclerosis.

The main objective of our study was to evaluate *C. pneumoniae* prevalence in Moroccan patients suffering from cardiovascular diseases. A total of 115 patients with cardiovascular diseases were enrolled, and their clinical and behavioral information was recorded. Blood was sampled from all patients as well as the atheroma plaques from 36 patients undergoing surgery. Nested PCR was performed for *C. pneumoniae* DNA detection in both peripheral blood mononuclear cells (PBMCs) and atheroma plaques. Statistical analysis was performed using EpiInfo software.

Data analysis showed cardiovascular disease dominance in men, with a sex ratio M/F of 3.4, a majority of tobacco users (52.2%), and many diabetics (44.3%). A significant difference between genders was shown for tobacco use ($p < 0.05$). Positive cases for PBMCs and atheroma plaques were 61% and 86%, respectively, and a significant difference between PBMCs and atheroma plaque infection was identified ($p = 0.02$). Data analysis also showed that 12% of patients presented only *C. pneumoniae* infection as a risk factor.

Therefore, the high prevalence of *C. pneumoniae* suggests its involvement in atherosclerosis, and further investigation is recommended for confirmation.

Introduction

Cardiovascular diseases are the leading cause of morbidity and mortality in industrialized and less-developed countries [1]. Genetic and environmental components, such as family history, hypercholesterolemia, tobacco and alcohol use, arterial hypertension, diabetes and obesity, explain much of the attributable risk for cardiovascular events [2,3], but many studies also suggest the involvement of various pathogens (bacteria and viruses) in the development of atherosclerosis. For example, *Chlamydia pneumoniae* (*C. pneumoniae*) was discussed in several studies [4–6].

C. pneumoniae is an obligate intracellular bacterium. It is Gram negative with binary multiplication and inter-human transmission. It is best known as a respiratory pathogen, causing 10% of community-acquired pneumonia, bronchitis and sinusitis [7,8]. Several studies showed that *C. pneumoniae* is involved in the development of cardiovascular diseases, resulting from atherosclerosis, which is a major public health problem worldwide [7–13]. Indeed, the rate of *C. pneumoniae* PCR positivity in atherosclerotic tissue samples can reach 60% [5,14–16].

In Morocco, data relating *C. pneumoniae* infection to atherosclerosis are scarce. One serological study on patients with cardiovascular diseases showed that *C. pneumoniae* seroprevalence reached 65.7% [17]. However, to confirm *C. pneumoniae* involvement in cardiovascular disease development, direct methods, such as culture and molecular detection, are the best diagnostic tools.

The main objective of our study was to investigate *C. pneumoniae* infection in Moroccan patients suffering from cardiovascular diseases and to evaluate its prevalence using molecular methods.

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Materials and methods

Patient sampling

A total of 115 patients attending for diagnostic and interventional coronary arteriography, including 86 men and 29 women (mean age 60 years), were enrolled between April 2013 and June 2015 at the cardiovascular surgery department at CHU Ibn Rochd, Casablanca, Morocco. They provided written informed consent to participate in the study before their information was recorded and samples obtained. Clinical records, including patient characteristics, clinical data and risk factors (diabetes, dyslipidemia, blood pressure, C-reactive protein (CRP), tobacco and alcohol use) were compiled for each patient, and blood samples (5 mL) were collected in ethylene diamine tetra acetic acid (EDTA) tubes. In parallel, 36 atheroma plaques were obtained from patients undergoing surgical intervention. Our study was approved by an institutional local ethics committee, and financial support was provided by the Institut Pasteur du Maroc, Casablanca, Morocco.

Peripheral blood mononuclear cell (PBMCs) separation

Immediately after collection, blood samples were diluted with an equal volume of minimal essential medium MEM (Gibco, Invitrogen, Grand Island, New York) and layered onto Ficoll (Pancoll, Biotech) for gradient separation of cells. The buffy coat layer was collected after centrifugation at 300 g for 30 min and then washed twice with MEM. PBMCs were resuspended in 1 mL MEM and stored at −80 °C until analysis.

DNA extraction from PBMCs

PBMCs were defrosted and a 500 μL cellular suspension was centrifuged and the pellet was treated with 500 μL lysis buffer (10 mM Tris HCl pH 8, 2 mM EDTA pH 8, and 40 mM NaCl, 0.5% SDS) supplemented with 10 mg/μL proteinase K (Invitrogen). The mixture was incubated overnight at 40 °C. Then, DNA was extracted using a phenol/chloroform method as previously described [18]. The lysate was sequentially treated with phenol and chloroform, and DNA was precipitated with 7.5 M ammonium acetate added to absolute ethanol, washed with 70% ethanol, dried under a laminar flow hood and then dissolved in 80 μL Tris EDTA buffer (10 mM, 1 mM, pH 8.0).

DNA extraction from atherosclerotic plaques

Approximately 100 mg of tissue samples were finely minced using scalpel blades and then placed in microtubes. A volume of 500 μL lysis buffer (100 mM NaCl, 10 mM Tris, HCl, pH 8, 25 mM EDTA, pH 8) supplemented with 0.5% SDS and proteinase K (10 mg/ml) was added. The mixture was incubated overnight at 50 °C. DNA was extracted by phenol/chloroform following the steps previously described.

2.3. C. pneumoniae *DNA amplification*

DNA detection in both PBMCs and atheroma plaques was performed by nested PCR using, in the first round, HL1: 5′-GTTGTTCATGAAGGCCTACT-3′ and HR1: 5′-TGCATAACCTACGGTGTGTT-3′ primers, which amplify a 437 bp in a pstI cloned gene fragment, and in the second round IN1: 5′-AGTTGAGCATATTCGTGAGG-3′ and IN2: 5′-TTTATTTCCGTGTCGTCCAG-3′ primers, to yield a 128 bp fragment [19]. For both rounds, DNA was amplified in 25 μL volumes containing 0.5 μM primers, 200 mM deoxyribonucleotides, 1X PCR buffer (Invitrogen), 2.5 mM $MgCl_2$, 1 U Taq polymerase (Invitrogen) and 2 μL DNA sample or first-round PCR product. Positive and negative controls were run in all PCR reactions. The PCR cycling conditions for both the first and the second rounds were as follows: one cycle at 95 °C for 5 min and 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, followed by strand elongation at 72 °C for 10 min in the thermocycler (S1000 ™Thermal Cycler, Biorad). The products were visualized by electrophoresis on 1.5% agarose gel stained with ethidium bromide.

Table 1
Risk factor distribution according to gender.

	Female (n = 29)	Male (n = 86)	Total (n = 115)	*P* value
Diabetes	15 (51%)	36 (42%)	51	0.355
High blood pressure	14 (48%)	26 (30%)	40	0.077
Tobacco use	1 (3.4%)	59 (69%)	60	0.000
Alcohol use	1 (3.4%)	12 (14%)	13	0.122
Dyslipidemia	10 (34%)	19 (22%)	29	0.121
High CRP level	3 (10%)	20 (23%)	23	0.01

Statistical analysis

All data were analyzed using Epi Info software. Qualitative variables and PCR results were compared by chi-square (X^2) test, and Fisher's exact test was used when the minimum estimated expected value was less than 5. A p value <0.05 was considered significant.

Results

Patient characteristics

The demographic data analysis showed a dominance of cardiovascular diseases in male patients with 78% of cases, and the M/F sex ratio was 3.4. Clinical risk factor analysis revealed that the majority of patients were tobacco users (52.2%), and diabetes and high blood pressure were observed in 44.3% and 34.7% of patients, respectively, whereas dyslipidemic patients represented 32.6% and alcohol users were 11.3% of patients. High CRP levels were observed in 76% of 30 recorded patients. When comparing the risk factors between males and females, the statistical analysis showed a significant difference only in tobacco use ($p < 0.0001$) and CRP ($p = 0.01$) (Table 1).

The cardiovascular pathology distribution showed a dominance of peripheral artery disease (54%), followed by myocardic ischemia (42%) and aortic stenosis (4%).

Molecular detection of C. pneumoniae *in PBMCs and atheroma plaques*

The nested PCR results revealed 71.3% (82/115) of positive cases in both PBMCs and/or atheroma plaques, with 78% (64/82) in males and 22% (18/82) in females (Fig. 1). *C. pneumoniae* distribution according to the specimen type (PBMCs and atheroma plaques) and gender showed that a total of 61% (70/115) of PBMC samples were positive, and men were more likely to have circulating *C. pneumoniae*, with 78.5%. In the atheroma plaques, *C. pneumoniae* was detected in 86% (31/36) of cases, and males and females represented 84% (26/31) and 16% (5/31), respectively (Table 2).

Atheroma plaque tissues were sampled in different vascular lesion locations. The PCR results showed that the femoral artery was most highly affected with 55% of positive cases (Table 3).

The pattern of *C. pneumoniae* distribution among 36 patients undergoing surgery showed 56% (20/36) of positive cases in both atheroma plaques and PBMCs; 28% (10/36) were positive in the atheroma plaques only, whereas one case (3%) was only positive

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