Chlamydia pneumoniae DNA in the Arterial Wall of Patients with Peripheral Vascular Disease

J. Gutiérrez, J. Linares-Palomino, C. Lopez-Espada, M. Rodríguez, E. Ros, G. Piédrola, M. del C. Maroto

Abstract

Background: *Chlamydia pneumoniae* is a human respiratory pathogen that has recently been related to the genesis of symptomatic atherosclerosis. *C. pneumoniae* has been studied more widely in relation to coronary atherosclerosis than to peripheral arterial occlusive disease (PAOD). The present study aimed to retrospectively analyze the presence of *C. pneumoniae* DNA in patients with PAOD.

Materials and Methods: A seminested PCR method was applied on 85 samples from 71 patients with PAOD secondary to surgical treatment. The control group comprised 50 patients with chronic superficial venous insufficiency who required varicose resection surgery.

Results: The number of patients, number of samples studied and percentage of patients found to be positive in the PCR study were 17, 18 and 59%, respectively, for arteries of the lower extremities; 15, 16 and 60% for aneurysm of the abdominal aorta; 22, 23 and 73% for carotid stenosis and 17, 18 and 65% for aortic stenosis. *C. pneumoniae* DNA was found in six external pudendal arteries (12%) of the control group, significantly lower than the incidence in the patient group ($p < 0.0001$).

Conclusion: A causal relationship between chronic *C. pneumoniae* infection and PAOD cannot be ruled out. On the contrary, the high incidence of *C. pneumoniae* DNA detected in our patients suggests that *C. pneumoniae* infection may play some role in the pathogenesis of peripheral vascular disease.

Key Words

DNA · *Chlamydia pneumoniae* · Atherosclerosis · PCR

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Introduction

Chlamydia pneumoniae is a human respiratory pathogen that has recently been related to the genesis of different types of disease, including pharyngitis, sinusitis, bronchitis, mild to severe pneumonia and asthma. Various studies linked *C. pneumoniae* infection to symptomatic atherosclerosis (AT), reporting higher levels of *C. pneumoniae* antibodies in patients with this disease versus controls [1–10]. Furthermore, some authors reported an increase in IgG antibodies prior to the onset of symptoms associated with the disease or after an atherectomy [10]. Nevertheless, other studies found no such relationship [11, 12]. Coronary atherosclerosis has been the most widely-studied disease in this respect, whereas the relationship between *C. pneumoniae* infection and peripheral arterial occlusive disease (PAOD) has received less attention.

Different methods can be utilized to analyze the relationship between *C. pneumoniae* and a clinical process. Culture of the bacteria is difficult and not very profitable. The PCR test is the most sensitive method and, in conjunction with bacteria culture, forms the gold standard diagnostic technique.

Studies on *C. pneumoniae* and PAOD are at the analytical stage and have produced conflicting conclusions depending on the author (Table 1) [13–29]. PAOD is a widespread and chronic disease and investigation into the DNA of a prevalent microorganism such as *C. pneumoniae* in the artery provides a way of establishing whether the agent may be implicated in the disease or, at least, of discovering the behavior of this biological parameter. Relationships between human atherosclerotic occlusive disease and herpes virus, cytomegalovirus, and *Helicobacter pylori* have been reported [2].

The aim of the present study was to retrospectively analyze, using PCR, the presence of *C. pneumoniae* DNA in patients with PAOD. Significant differences compared with the control population would either support or rule out the hypothesis of a relationship between *C. pneumoniae* and this disease.

J. Gutiérrez *(corresponding author)*, **G. Piédrola, Maria del Carmen Maroto**
Dept. of Microbiology, University Hospital San Cecilio, University of Granada, Avda. de Madrid 12, E-18012 Granada, Spain;
Phone: (+34/62) 6761948, Fax: (+34/95) 8246119,
e-mail: josegf@goliat.ugr.es, joseg.f@wanadoo.es
J. Linares-Palomino, Cristina Lopez-Espada, E. Ros
Dept. of Surgery, University Hospital San Cecilio, University of Granada, Granada, Spain
M. Rodríguez
Dept. of Microbiology, University of Cadiz, Spain

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Materials and Methods

PCR was used to detect *C. pneumoniae* DNA in the biopsy specimens of blood vessels obtained between September 1998 and December 1999 from patients treated at the Vascular Surgery Department of San Cecilio University Hospital. The processing and interpretation of all the laboratory determinations were performed blindly.

Patients

We studied 85 samples (Table 2) from 71 patients (Table 3) with severe atherosclerosis who underwent surgical treatment. In 15 cases samples were taken from two areas with atherosclerotic disease and in three cases samples were taken from three areas. From the remaining 47 patients a specimen was taken from only one area, which was always diseased (atherosclerosis or aneurysm). In cases of aneurysm of the abdominal aorta (AAA), samples of the arterial wall were collected and also, in ten cases, simultaneous samples of non-wall tissue that formed the aneurysmal thrombus. In the carotid stenosis group, two samples were gathered from one patient, one from each carotid, with an interval of 6 months between the operations, during which the patient was treated with platelet anti-aggregants and clarithromycin at doses of 500 mg/8 h for 2 months. Finally, we considered biopsy specimens of arterial wall tissue from aortic and iliac arterial stenoses.

Table 1
Results obtained by different authors in studies on *C. pneumoniae* DNA in peripheral arteries.

Author	Localization of the atherosclerotic plaque sample	Positive (%)
Blasi et al. [13]	AAA	49
Campbell et al. [14]	Carotid endarterectomy	53
Grayston et al. [15]	Carotid endarterectomy	57
Jackson et al. [16]	Carotid endarterectomy	24
Jantos et al. [17]	Carotid endarterectomy	8
Juvonen et al. [18]	AAA	100
Kuo et al. [19]	Popliteal; femoral	19; 5
Karlsson et al. [20]	AAA	35
Lindholt et al. [21]	AAA	0
Maass et al. [22]	Carotid endarterectomy	15
Maass et al. [23]	Carotid; aorta; iliac	15; 18; 15
Meijer et al. [24]	AAA	0
Ong et al. [25]	AAA	44
Ouchi et al. [26]	Iliac	30
Paterson et al. [27]	Carotid endarterectomy; carotid of cadaver	0; 0
Petersen et al. [28]	AAA	35
Wadrchal et al. [29]	Carotid endarterectomy; AAA	28; 21

AAA: aneurysm of abdominal aorta

Controls

The control group comprised 50 patients with chronic superficial venous insufficiency who required the surgical excision of varicose veins. They were selected for their age (over 55 years) and to match the gender distribution of the patient group (Table 3). They all underwent clinical examination, electrocardiography and carotid echo-Doppler to rule out the presence of known AT (coronary, cerebrovascular, or peripheral). All of the patients and controls in the study gave their informed written consent and the study was approved by the hospital ethics committee.

All of the patients received a prophylactic iv dose of 1 g cefazolin. Patients allergic to cefazolin were scheduled to receive erythromycin, although this was never necessary in the present study. None of the patients had active infections that required antibiotic therapy before the surgery.

The biopsy specimens were obtained in the patient group from atherosclerotic plaques extracted during reconstructive vascular surgery (aneurysms) or during revascularization surgery with bypass or thromboendarterectomy (remaining groups). In the control group, 1 cm of the external pudenda artery was obtained during the varicose vein surgery. All of the biopsy specimens were immediately washed with physiologic serum and prepared in the transport medium and sterile sucrose–phosphate-glutamate buffer. They were stored at between –4 °C and –10 °C for 12–16 h and then maintained at –70 °C prior to PCR testing for the detection of *C. pneumoniae* DNA.

Determination of *C. pneumoniae* DNA in Biopsies

The High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Germany) was used. We performed a seminested PCR based on the method of *Campbell* et al. [14], which uses two sets of primers to detect a DNA fragment (Pst1) of *C. pneumoniae*. 2 µl of extracted DNA was amplified in a total volume of 100 µl. The final mixture of the first PCR (PCR Core Kit, Roche Molecular Biochemicals) contained 0.5 µM primers (HL-1: 5'GTTGTT-CATGAAGGCCTACT3'; HR-1: 5'TGCATAACC-TACGGTGTGTT3'), 50 mM deoxynucleoside triphosphates

Table 2
Results of the PCR test for *C. pneumoniae* DNA in patients with systemic atherosclerosis secondary to peripheral vascular surgery.

Group	Patients n	Samples n	Positive patients (%)	Localization of sample	PCR result (% positive)
Arteries of lower extremity	17	18	59	17 common femoral 1 popliteal aneurysm	8+ (59%) 0+ (0%)
Abdominal aortic aneurysm	15	26	60	16 wall 10 thrombus	9+ (56%) 2+, 4– , 4 not assessable
Carotid stenosis	22	23	73	Endarteries	17+ (74%)
Aortic stenosis	17	18	65	12 infrarenal aorta 6 iliac	10+ (83%) 2+ (33%)

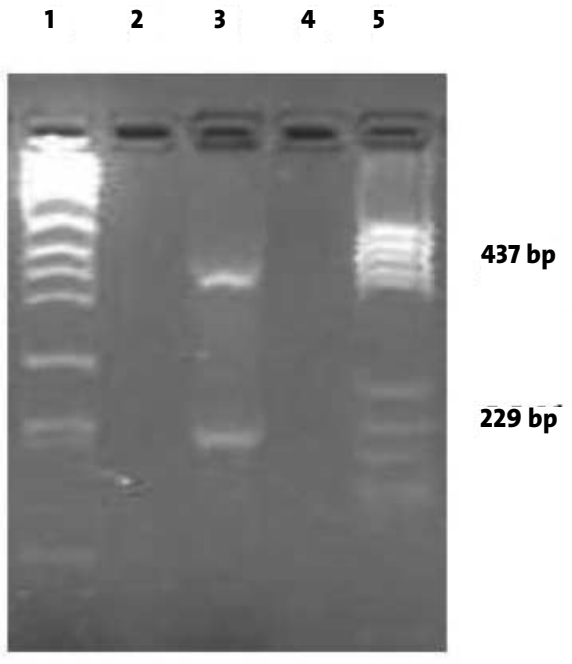

Figure 1. PCR results in positive and negative controls. Amplification of *Chlamydia pneumoniae* DNA. Lanes: MW 154-2176 bp (lane 1), negative control (lane 2), positive sample (437 bp and 229 bp) (lane 3), negative sample (lane 4), MW 8–587 bp (lane 5).

(dNTPs), 1 × PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl), 5 mM $MgCl_2$ and 1 U of *Taq* polymerase. A Perkin-Elmer 9600 thermocycler was used for the PCR under the following conditions: after an initial 4 min denaturation step at 94 ºC, 40 amplification cycles were performed, each consisting of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 1 min and primer extension at 72 ºC for 1 min. Amplification was completed with a final incubation step at 72 ºC for 7–10 min. 2 µl of the first PCR reaction was amplified in 100 µl final volume (PCR Core Kit) using 0.5 µM primers (HM-1: 5'GTGTCATTCGCCAAGGTT3'; HR-1: 5'TGCATAACCTACGGTGTGTT3'), 200 µM dNTPs, 1 × PCR buffer, 3 mM $MgCl_2$ and 1 U of *Taq* polymerase. Amplification conditions were as described for the first PCR, except that annealing was performed at 48 ºC. PCR products (20 µl) were analyzed by electrophoresis in 2% agarose gels separated at 110 V for 1 h by using Tris-borate-EDTA buffer (pH 8.3). Nucleic acids were then visualized by staining on ethidium bromide-stained gels (0.5 µg/ml). The expected amplification products of the HL-1/HR-1 primer pair and the HM-1/HR-1 primer pair were 437 and 229 bp, respectively. Samples that presented amplified fragments of 229 bp ± 437 bp in size were considered to be positive. During the first amplification, primers of the human beta-actin gene were added in order to determine whether there were PCR inhibitors and whether the DNA had been correctly extracted from the samples (Figure 1). These primers gave rise to a 331 bp fragment.

To avoid false-positive amplifications, procedures recommended to prevent contamination were strictly observed [30] and all of the reactions were performed under stringent conditions. All reagents were aliquoted and stored in distinct locations. PCR reagents were prepared before each assay in a master mixture which was then aliquoted. The preparation of the master mixture, the extraction of the DNA and addition of the template to the PCR mixture and the thermal cycling were performed in three different, well-separated rooms, each with its own dedicated set of micropipettes and gowns. Only aerosol-resistant barrier pipette tips were used. Meticulous laboratory techniques and adherence to standard PCR anti-contamination procedures were the norm, including frequent glove changes and decontamination of surfaces with UV light and sodium hypochlorite. All tubes, pipette tips and reagents, except for the primers and *Taq* polymerase, were exposed to 254 nm of UV light in a nucleic acid linker oven (Stratalinker UV Crosslinker, Stratagene) before use. A number of negative (numerous negative water and PCR reagent-only samples) controls were included in each PCR assay.

Table 3
Characteristics of study populations.

Characteristics	Patients	Controls	p
Age (years)	67 ± 8	60 ± 3	< 0.0001 [a]
Age > 60 years (%)	76.9	56	0.073 [b]
Male (%)	73.1	80	0.492 [b]

[a] Student's t-test; [b] Chi-square test

Statistics

The statistical analysis used the SPSS statistical package (SPSS Inc. Version 9.0, 1999). The continuous variables were compared with the Student's t-test when normality was assured. The discrete variables were analyzed with the Chi-square test.

Results

The seminested PCR protocol detected *C. pneumoniae*-specific DNA sequences in 48 of the 85 (56.47%) atherosclerotic samples.

Table 2 shows the results obtained in the different groups of patients. Out of the 17 samples from the common femoral artery, eight were positive. The sample from the popliteal aneurysm was negative, whereas a sample from the wall of the aortic aneurysm of the same patient was positive.

In most of the samples from AAA, the PCR testing of the thrombus gave negative results or could not be assessed because of the non-amplification of the beta-actin gene. In only two cases were these thrombus samples positive. Out of 16 samples from the AAA wall, nine were positive; among the seven negative samples were two thoracoabdominal aneurysms.

There was a high incidence of positive results in the samples from the carotid artery. In the patient who provided one sample from each carotid, the first was positive whereas the second, after the antibiotic treatment, was negative.

A very high proportion of stenosed aorta samples (always very diseased) were positive (10/12), in contrast to the results for the iliac artery.

Moreover, *C. pneumoniae* DNA was found in six external pudendal arteries of the control group (12%), sig-

nificantly less frequently than in the patient group ($p < 0.0001$).

Discussion

The findings of our case-control study support the role of chronic *C. pneumoniae* infection in PAOD, because significantly more DNA of this pathogen was detected in samples from patients with this disease compared with control samples. Our study differed in two respects from previous publications on this issue (Table 1); our series included a large number of samples from different anatomic sites, none of which were cardiac in origin (the strong relationship of *C. pneumoniae* with coronary disease sites has been widely demonstrated) and we used a seminested PCR method.

It was not easy to form a control group because of the difficulty of finding elderly men whom we could reasonably ask to have part of an artery (the external pudendal in our case) extracted during a surgical procedure. The control group was selected after the cases had been recruited, in order to control for age and gender, two risk factors in AT. The patients with PAOD were significantly older than the controls, with a 7-year age difference. However, we believe that this difference had little effect on our results, because the mean age of each group was at least 60 years and the proportion of over 60-year-olds was similar (Table 3). The groups were matched for gender, proportion of over 60-year-olds, tobacco use and social-health status.

The extraction of a segment of the external pudenda artery is a reasonable request during the conventional surgery of varicose veins, because this artery can be ligated if necessary. The present study was the first to extract a 1 cm segment of this artery for analysis, so that diseased and healthy arteries could be compared between the two matched groups. There were no postoperative complications attributable to the extraction of this biopsy specimen among the patients undergoing varicose vein surgery (control group). We were able to use this segment as a control to avoid selection bias by comparing arteries that normally suffer AT (carotids, etc.) with those that are rarely affected (pudendal). We think that the artery of a live person always serves as a better witness than does that of a cadaver, used in most of the published studies [27, 31]. Finally, the control groups used in many earlier studies were not adequate, because they comprised samples from adults of very different ages who were not matched to the cases.

PCR is a highly specific technique which provides an extremely reliable diagnosis [14, 22, 23]. We applied a seminested PCR test in an attempt to increase the sensitivity and specificity of the detection and reduce the number of inhibited samples compared with the original method of *Campbell* et al. [14] which used simple PCR, although some of our samples of thrombotic material from AAA showed inhibition.

The highest percentage of positive samples from AAA cases came from the arterial wall. This may be because aneurysmal disease is a distinct pathologic entity from AT, as proposed by some authors [32]. In fact, it is uncommon to find atherosclerotic and aneurysmal disease in the same patient. This may account for the smaller number of positive findings in this group of our series. Out of the seven negative samples, two were from thoracoabdominal aneurysms, which are not considered of atherosclerotic origin but rather caused by the degeneration of the mid-layer of the aorta [32].

A high proportion of the present samples from the carotid artery were positive, even somewhat higher than the 15–61% range previously reported, [15, 16, 22, 23, 29]. Two earlier studies found no association between *C. pneumoniae* and carotid AT: one found no DNA [27] and the other did not consider the association significant, because of the low percentage (8%) of positive cases [17]. We also obtained higher proportions of positive samples from the lower extremity arteries and aortic stenoses than did other authors (Table 1).

Our positive findings in the control samples should not be considered exceptional, because *C. pneumoniae* has been detected in other areas where AT is not usually present, such as the saphenous vein and the internal mammary artery [29, 33].

On the basis of the present results, a causal relationship between chronic *C. pneumoniae* infection and PAOD cannot be ruled out. On the contrary, the high prevalence of the DNA in the patients suggests that the infection may play some role in the pathogenesis of disease.

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