CHLAMYDIA PNEUMONIAE IN ASYMPTOMATIC CAROTID ATHEROSCLEROSIS

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Received March 15, 2005 – Accepted April 7, 2005

We evaluated, in 415 patients with asymptomatic carotid atherosclerosis: (i) the prevalence of *C. pneumoniae* DNA in atherosclerotic carotid plaques and peripheral blood mononuclear cells (PBMC); (ii) the distribution of *C. pneumoniae* in atherosclerotic carotid plaques and PBMC from the same patients; (iii) the correlation between circulating anti-chlamydial antibodies and the presence of *C. pneumoniae* DNA. Overall, 160 atherosclerotic carotid plaques and 174 PBMC specimens from patients with asymptomatic carotid atherosclerosis were examined by *ompA* nested touchdown PCR for presence of *C. pneumoniae*. In addition, *C. pneumoniae* DNA was detected in 81 specimens of atherosclerotic carotid plaque and PBMC obtained from the same patients. *C. pneumoniae* DNA was found in 36.9% of atherosclerotic carotid plaques and in 40.2% of PBMC specimens examined (P=NS). With regard to 81 patients, *C. pneumoniae* DNA was detected in 27.2% of atherosclerotic carotid plaques and in 44.4% of PBMC specimens (P=0.05). In 18 patients, the presence of *C. pneumoniae* DNA in PBMC specimens and atherosclerotic carotid plaques coincided (P=0.005). No statistically significant association was found between anti-*C. pneumoniae* antibodies (IgG and IgA) and positive PCR results. In conclusion, our results suggest that the detection of *C. pneumoniae* DNA in PBMC specimens seems to be a first-choice method to identify the patients at risk for endovascular chlamydial infection.

A number of infectious agents have been implicated in atherosclerosis, such as *Helicobacter pylori*, Cytomegalovirus and periodontal bacteria, but by far the most studied is *Chlamydia pneumoniae* (1). *C. pneumoniae*, a common pathogen in human respiratory tract infections, is the sole viable pathogen detected in atherosclerotic plaques arteries (2-4). During the past decade the role of this organism in the development of atherosclerosis and coronary heart disease has been extensively explored and association with other vascular diseases such as atherosclerotic carotid diseases and ischemic cerebrovascular diseases has been proposed (5).

The first suggestion that *C. pneumoniae* may be associated with atherosclerosis was proposed in 1988 by Saikku et al. (6). Similar seroepidemiological results have now been confirmed by about 38 studies of varying designs (retrospective, cross-sectional, case-control, or prospective) world wide (7-11). However, reports from several studies (12-14) have failed to demonstrate an association between

Key words: Chlamydia pneumoniae, atherosclerotic carotid disease, polymerase chain reaction

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0394-6320 (2006)

antibodies to *C. pneumoniae* and atherosclerosis since a large part of the population has pre-existing IgG antibodies from previous exposure(s). On the other hand, the detection of *C. pneumoniae* DNA in atherosclerotic lesions of subjects with cardiovascular diseases has strengthened the likelihood of its possible involvement in the pathogenesis of atherosclerosis.

Evidence for the presence of the organism in the atherosclerotic lesions has emerged from more than 40 studies using immunohistochemistry, electron microscopy and amplification of chlamydial DNA by polymerase chain reaction (PCR) (8, 15-17). Recently several studies have shown the presence of *C. pneumoniae* DNA in peripheral blood mononuclear cells (PBMC) of patients with atherosclerotic cardiovascular disease, suggesting that detection of *C. pneumoniae* DNA in PBMC may be a valid surrogate marker to identify individual risk for endovascular chlamydial infection (18-21).

The aims of our study were to investigate, in patients with asymptomatic carotid atherosclerosis: (i) the prevalence of *C. pneumoniae* DNA in atherosclerotic carotid plaques and peripheral blood mononuclear cells (PBMC); (ii) the distribution of *C. pneumoniae* in atherosclerotic carotid plaques and PBMC from the same patients; (iii) the correlation between circulating anti-chlamydial antibodies and the presence of *C. pneumoniae* DNA.

MATERIALS AND METHODS

Between January 2003 and December 2004, 415 patients (303 male and 112 female; mean age 72±11 years) with asymptomatic carotid atherosclerosis were enrolled. All patients underwent colour flow echo Doppler imaging, and carotid angiography. One hundred and sixty specimens of atherosclerotic carotid plaque were obtained from 160 patients during carotid endarterectomy. One hundred and seventy-four PBMC specimens were obtained from 174 patients who did not undergo a carotid endarterectomy. In addition, 81 patients (61 male and 20 female, mean age 73±12 years) with asymptomatic carotid atherosclerosis were included in this study; specimens of atherosclerotic carotid plaque and PBMC were obtained from each patient during carotid endarterectomy. Endarterectomy involved a long arteriotomy with dissection of the atherosclerotic lesions in toto. The degree of carotid artery stenosis in patients undergoing carotid endarterectomy was greater than 85%.

For serologic analysis, a serum sample was obtained from each patient enrolled in this study.

Patients characteristics were as follows: 304 (73.2 %) were current or past-smokers, 161 (38.8 %) had diabetes, 311 (74.9%) had hypertension, 149 (35.9%) had dyslipidemia, 199 (47.9%) had coronary heart disease.

DNA isolation

Carotid plaques. Multiple sections of each atherosclerotic carotid plaque, stored at –80°C, were analyzed as described recently (22). DNA from atherosclerotic carotid plaque was extracted by using a QIAamp DNeasy Tissue kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in a final volume of 100 µl, aliquoted, and stored at –20°C.

PBMC. Blood samples (5 ml) were processed to isolate PBMC in accordance with a method described by Condos et al. (23), and stored at –80°C.

DNA from PBMC specimens was extracted by using a QIAamp DNA Mini-kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in a final volume of 100 µl, aliquoted, and stored at –20°C.

In order to minimise the risk of false-positive results, negative reagent controls obtained by replacing clinical specimens with an equal volume of ultrapure water PCR grade were included and processed throughout the whole extraction procedure.

Polymerase chain reaction

In order to assess the presence of possible PCR-inhibitors human β-globin gene was amplified from all DNA samples. Detection of *C. pneumoniae* DNA was performed by *ompA* nested touchdown PCR as previously described (24). All amplification reactions were carried out in a total volume of 50 µl containing 5 µl of extracted DNA.

Four controls, consisting of one positive and three negative controls (*C. pneumoniae* AR-39 and ultrapure water PCR grade, respectively), and negative extraction controls (as previously described) were also included. All negative controls (extraction and PCR controls) were exposed to air throughout specimen addition.

Each clinical specimen was analyzed in replicates of 3. A specimen was considered positive if two of the three replicates were positive. PCR-positive specimens were also amplified in triplicate by non-nested PCR targeting the *C. pneumoniae* specific *PstI* fragment (25). To prevent contamination of the samples, reagent preparation and product analysis were performed in three separate areas with the use of filter-tipped pipets.

Serologic analysis

C. pneumoniae antibodies (IgG, IgA) were measured by a microimmunofluorescence test. Titers of IgG 1:32 or higher and IgA 1:16 or higher were considered positive.

Statistical analysis

The results were subjected to statistical analysis, using the χ^2 test to compare frequency distributions, with Yates' correction. When the minimum estimated expected value was <5, Fisher's exact test was used. Furthermore, McNemar's test was used for comparisons of paired results. Statistical significance was determined at an alpha-level of 0.05.

RESULTS

PCR detection of C. pneumoniae

Overall, 160 atherosclerotic carotid plaques and 174 PBMC specimens from patients with asymptomatic carotid atherosclerosis were examined for presence of *C. pneumoniae.*

C. *pneumoniae* DNA was detected in 59 (36.9%) of 160 atherosclerotic carotid plaques and in 70 (40.2%) of 174 PBMC specimens examined (P=not significant) (Fig. 1). Prevalence of *C pneumoniae* DNA in atherosclerotic carotid plaques and PBMC obtained from each of 81 patients with asymptomatic carotid atherosclerosis is shown in Fig. 2. A higher positivity rate of *C. pneumoniae* was found in PBMC (44.4%) than in atherosclerotic carotid plaques (27.2%) (p=0.05).

The pattern of distribution of *C. pneumoniae* among 81 patients is shown in Table I. *C pneumoniae* DNA was detected in both atherosclerotic carotid plaques and PBMC in 18 patients, in PBMC but not in atherosclerotic carotid plaques in 17 patients, and in atherosclerotic carotid plaques in only 4 patients. Eighteen patients were negative by both *C. pneumoniae* serology and PCR in atherosclerotic carotid plaques and PBMC.

Table I. *Pattern of distribution of C. pneumoniae among 81 patients with asymptomatic carotid atherosclerosis.*

PBMC	*Carotid plaques*	*Serology findings*	*No. of patients*
Positive	Positive	Positive	18
Positive	Negative	Positive	17
Negative	Positive	Positive	4
Negative	Negative	Positive	24
Negative	Negative	Negative	18

* *Defined as IgG ≥32 or IgA ≥16.*

Serologic detection of C. pneumoniae

C. pneumoniae IgG and IgA were found in 73.9% (247/334) and in 47.3% (158/334) of patients with

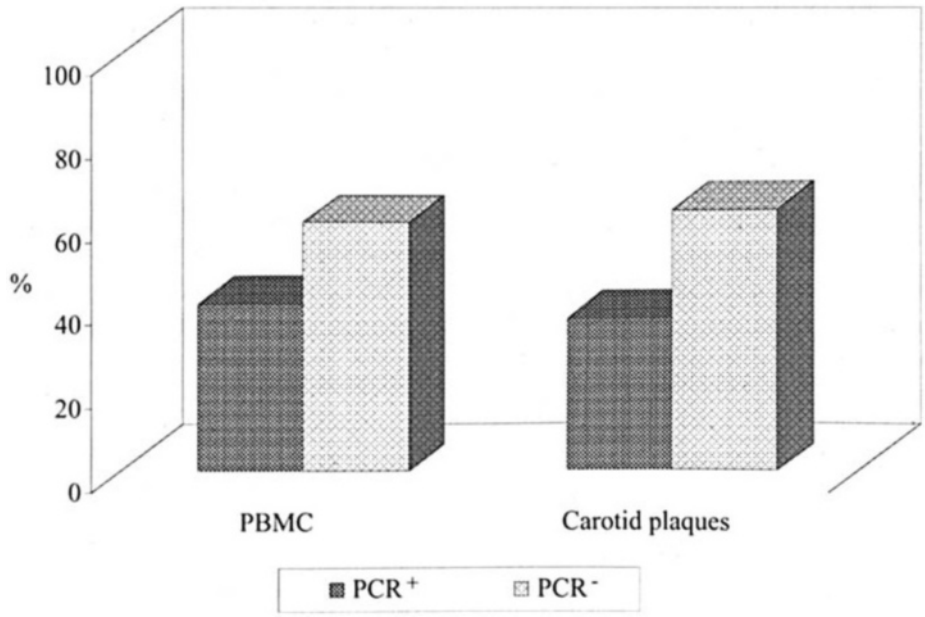

Fig. 1. *Prevalence of Chlamydia pneumoniae DNA, detected by ompA nested touchdown PCR, in PBMC and atherosclerotic carotid plaques of patients with asymptomatic carotid atherosclerosis (PCR+, Polymerase chain reaction positive; PCR-, Polymerase chain reaction negative).*

Table II. *Results of Chlamydia pneumoniae DNA in relation to IgG and IgA anti-Chlamydia pneumoniae in patients with asymptomatic carotid atherosclerosis.*

	PBMC			P	Carotid plaques			P
	PCR⁺ *(n=70)*	PCR⁻*(n=104)*	Total *(n=174)*		PCR⁺*(n=59)*	PCR⁻ *(n=101)*	Total *(n=160)*	
IgG ≥32	54 (77.1%)	76 (73.1%)	130 (74.7%)	NS	48 (81.3%)	69 (68.3%)	117 (73.1%)	NS
IgG ≥512	31 (44.3%)	37 (35.6%)	68 (39.1%)	NS	26 (44.1%)	38 (37.6%)	64 (40.0%)	NS
IgA ≥16	43 (61.4%)	51 (49.0%)	95 (54.6%)	NS	28 (47.4%)	35 (34.6%)	63 (39.4%)	NS
IgA ≥256	17 (24.3%)	20 (19.2%)	37 (21.3%)	NS	17 (28.8%)	16 (15.8%)	33 (20.6%)	NS

PCR⁺= polymerase chain reaction positive; PCR⁻= polymerase chain reaction negative.

asymptomatic carotid atherosclerosis. High IgG (≥512) and IgA (≥256) titers were found in 39.5% (132/334) and in 20.9% (70/334) respectively of patients examined.

As regard to patients whose specimens of atherosclerotic carotid plaques and PBMC specimens were analyzed, *C. pneumoniae* IgG and IgA were found in 75.3% (61/81) and in 48.1% (39/81) respectively of 81 patients; high IgG (≥512) and IgA (≥256) titers were found in 35.8% (29/81) and in 22.2% (18/81), respectively.

PCR findings in atherosclerotic carotid plaques, and in PBMC specimens are compared with *C. pneumoniae* IgG and IgA antibodies in Tables II and III. No statistically significant association was found between anti-*C. pneumoniae* antibody titers and positive PCR results reported for 334 patients with asymptomatic carotid atherosclerosis. The same finding was observed when we examined anti-*C. pneumoniae* antibody titers and positive PCR results reported for 81 patients.

DISCUSSION

Our study provides further evidence for the involvement of *C. pneumoniae* in carotid atherosclerosis. Firstly, *C. pneumoniae* is able to disseminate systemically from the lungs through infected PBMC and to localize in arteries, where it may infect endothelial cells, vascular smooth muscle cells, monocytes/macrophages and promote inflammatory atherogenous process. Indeed, in our study, *C. pneumoniae* DNA was found in PBMC (40.2%) as well as in atherosclerotic carotid plaques (36.9%).

In the literature, reported PCR detection rates of *C. pneumoniae* DNA in PBMC and in atherosclerotic carotid plaques from patients with atherosclerotic cardiovascular diseases vary between 6% (26) and 87% (27) and between 0% (28) and 83% (27) respectively. However, no standardized PCR assays are available yet and the discordant positivity rates may be attributable to low concentration of *C. pneumoniae* DNA in clinical

Table III. *Results of Chlamydia pneumoniae DNA in relation to IgG and IgA anti-Chlamydia pneumoniae in 81 patients with asymptomatic carotid atherosclerosis.*

	PBMC			P	Carotid plaques			P
	PCR⁺ *(n=35)*	PCR⁻ *(n=46)*	Total *(n=81)*		PCR⁺ *(n=22)*	PCR⁻ *(n=59)*	Total *(n=81)*	
IgG ≥32	28 (80.0%)	33 (71.2%)	61 (75.3%)	NS	18 (81.8%)	43 (72.9%)	61 (75.3%)	NS
IgG ≥512	13 (37.1%)	16 (34.8%)	29 (35.8%)	NS	9 (40.9%)	20 (33.8%)	29 (35.8%)	NS
IgA ≥16	18 (51.4%)	21 (45.6%)	39 (48.1%)	NS	11 (50.0 %)	28 (47.4%)	39 (48.1%)	NS
IgA ≥256	7 (20.0%)	11 (23.9%)	18 (22.2%)	NS	5 (22.7%)	17 (28.8%)	18 (22.2%)	NS

PCR⁺= polymerase chain reaction positive; PCR⁻= polymerase chain reaction negative.

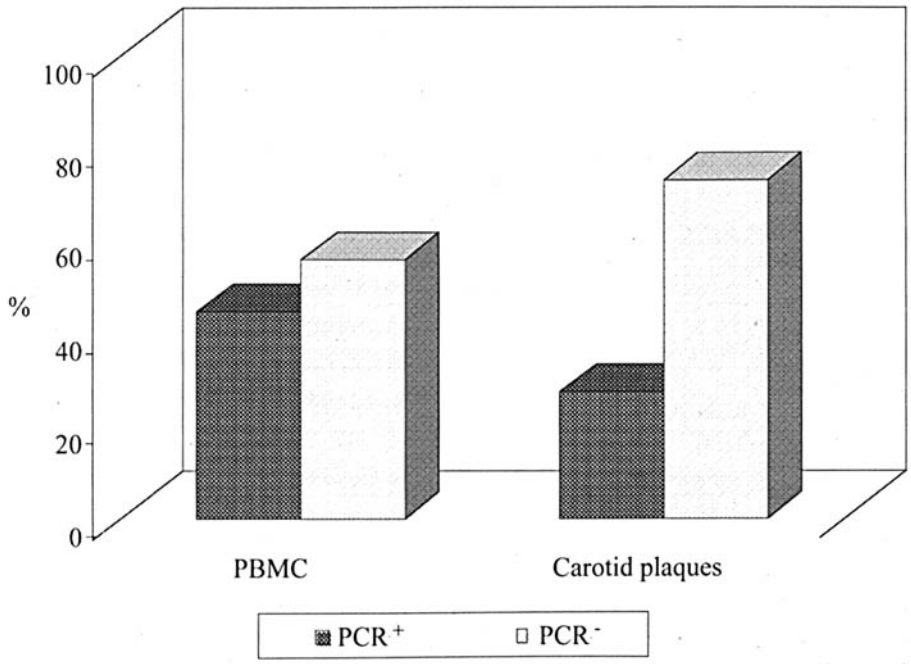

Fig. 2. *Prevalence of Chlamydia pneumoniae DNA, detected by ompA nested touchdown, PCR in PBMC and atherosclerotic carotid plaques of 81 patients with asymptomatic carotid atherosclerosis (PCR+, Polymerase chain reaction positive; PCR-, Polymerase chain reaction negative).*

specimens and, hence, to poor reproducibility of PCR results. Secondly, we examined atherosclerotic carotid plaques and PBMC obtained from each of 81 patients with asymptomatic carotid diseases for the presence of *C. pneumoniae* DNA. A significantly higher prevalence of *C. pneumoniae* DNA was found in PBMC (44.4%) than in atherosclerotic carotid plaques (27.2%) (P=0.05). In particular, in 18 patients the presence of C. *pneumoniae* DNA in PBMC specimens and atherosclerotic carotid plaques coincided (P=0.005). These results supported that detection of *C. pneumoniae* DNA in PBMC may be an alternative approach to identifying subjects carrying *C. pneumoniae* in the vascular wall. Indeed, in a previous study, we have demonstrated that *C. pneumoniae* DNA in PBMC may be associated with symptomatic carotid atherosclerotic disease (29).

However, to date, only a few reports (27, 29-31) on the presence of *C. pneumoniae* in atherosclerotic lesions and PBMC obtained from the same patients have been generated; in these studies *C. pneumoniae* was more common in the PBMC than in atherosclerotic lesions although this was not statistically significant. A similar study was carried out by Apfalter et al. (32). They have demonstrated, by PCR real-time, the presence of *C. pneumoniae* DNA in PBMC (5.6%) but not in atherosclerotic carotid plaques. A higher positivity rate of *C. pneumoniae* found in PBMC than in atherosclerotic carotid plaques may be explained by the patchy distribution of *C. pneumoniae* within atherosclerotic lesions. Furthermore, the detection of C. *pneumoniae* DNA in PBMC has the advantage that the blood specimen is easily available compared with atherosclerotic carotid plaque. Indeed, the atherosclerotic arteries plaques are obviously obtained too late in the course of the disease to be of clinical use. Therefore, the detection of C. *pneumoniae* DNA in PBMC seems to be a first-choice method to evaluate the role of C. *pneumoniae* in patients at risk of endovascular chlamydial infection. When evaluating IgG and IgA antibodies against C. *pneumoniae*, we, in accordance with other authors (27, 33-34), did not observe any statistically significant correlation between PCR detection of *C.*

pneumoniae and serology. These results confirmed that serological tests do not appear suitable for predicting vascular *C. pneumoniae* infection. Regarding the poor reproducibility of PCR results, we found (35), in accordance with the results of Smieja et al. (36), that repeated testing of the same specimen increased reproducibility of the PCR results, providing a more accurate estimate of the prevalence of *C. pneumoniae* in PBMC. However, the replicate PCR testing requires a more complex workflow and a careful control of product carryover contamination and is a labour-intensive procedure for routine clinical laboratories. These problems may be overcome with application of the quantitative real-time PCR technology which combines rapid amplification and sequence-specific detection of amplicons in an automated and standardized format.

In conclusion, we believe that development of a PBMC-based real-time PCR may be a promising future diagnostic approach to detect *C. pneumoniae* at low concentration in the circulation and in the vascular wall even if further studies are needed to evaluate whether the quantitative detection of *C. pneumoniae* could be of help in clarifying the etiology-pathogenic role of *C. pneumoniae* in patients at risk of atherosclerotic carotid disease.

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