

Research article

## Non-detection of *Chlamydia* species in carotid atheroma using generic primers by nested PCR in a population with a high prevalence of *Chlamydia pneumoniae* antibody

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Published: 24 August 2001

Received: 1 August 2001

BMC Infectious Diseases 2001, 1:12

Accepted: 24 August 2001

This article is available from: <http://www.biomedcentral.com/1471-2334/1/12>

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### Abstract

**Background:** The association of *Chlamydia pneumoniae* with atherosclerosis is controversial. We investigated the presence of *C. pneumoniae* and other *Chlamydia* spp. in atheromatous carotid artery tissue.

**Methods:** Forty elective carotid endarterectomy patients were recruited (27 males, mean age 65 and 13 females mean age 68), 4 had bilateral carotid endarterectomies (n= 44 endarterectomy specimens). Control specimens were taken from macroscopically normal carotid artery adjacent to the atheromatous lesions (internal controls), except in 8 cases where normal carotid arteries from post mortem (external controls) were used. Three case-control pairs were excluded when the HLA DRB gene failed to amplify from the DNA. Genus specific primers to the major outer membrane protein (MOMP) gene were used in a nested polymerase chain reaction (nPCR) in 41 atheromatous carotid specimens and paired controls. PCR inhibition was monitored by spiking with target *C. trachomatis*. Atheroma severity was graded histologically. Plasma samples were tested by microimmunofluorescence (MIF) for antibodies to *C. pneumoniae*, *C. trachomatis* and *C. psittaci* and the corresponding white cells were tested for *Chlamydia* spp. by nPCR.

**Results:** *C. pneumoniae* was not detected in any carotid specimen. Twenty-five of 38 (66%) plasma specimens were positive for *C. pneumoniae* IgG, 2/38 (5%) for *C. trachomatis* IgG and 1/38 (3%) for *C. psittaci* IgG.

**Conclusions:** We were unable to show an association between the presence of *Chlamydia* spp. and atheroma in carotid arteries in the presence of a high seroprevalence of *C. pneumoniae* antibodies in Northern Ireland.

## Background

*C. pneumoniae* is a respiratory pathogen causing community-acquired pneumonia, bronchitis, pharyngitis and sinusitis [1]. While an association between *C. pneumoniae* and atherosclerosis has been suggested, its exact role in the pathogenesis of atherosclerosis remains uncertain. Some, but not all, seroepidemiological studies [2],[3] have shown elevated antibody levels to *C. pneumoniae* in patients with coronary artery disease and many studies have also detected the presence of *C. pneumoniae* in atherosclerotic tissue [4],[5]. However, others have either failed to detect the organism [6] or demonstrated a low prevalence in arterial tissues [7].

The fact that both *C. psittaci* and *C. pneumoniae* are respiratory pathogens and that the pathogenesis of *C. pneumoniae* and atherosclerosis may be analogous to the development of trachoma by *C. trachomatis* [8], would support the necessity to determine whether other *Chlamydia* spp. beside *C. pneumoniae* are present in arterial tissue. In this study we aimed to investigate the presence of *Chlamydia* spp. in carotid artery atheroma using genus specific chlamydia primers in a nested polymerase chain reaction (nPCR).

## Materials and Methods

### Patients and specimens

Specimens were collected from 40 patients enrolled for elective carotid endarterectomy between 1998 and 1999. Patients' ages ranged from 42 to 84 years and included 27 males (mean age 65) and 13 females (mean age 68). Four patients (2 males and 2 females) had bilateral carotid endarterectomies giving a total of 44 endarterectomy specimens. All specimens were graded macroscopically as follows: Grade 0 normal artery, Grade 1 fatty streak, Grade 2 fibrolipid plaque and Grade 3 advanced lesion with fibrosis, calcification, ulceration or haemorrhage [9].

For each specimen macroscopically normal artery adjacent to the atheromatous area, removed as part of the normal surgical procedure, was used as an internal control (36 specimens). Endarterectomy specimens which did not have macroscopically normal areas adjacent to the atheromatous section were age and sex matched with external controls taken from normal post-mortem carotid arteries (8 specimens) following consent from relatives of the deceased. The carotid arteries were collected immediately from theatre and divided into two main sections, atheromatous and non-atheromatous, using a sterile technique. Each section was further divided to obtain specimens for histology, which were stored in 10% non-buffered formalin. Specimens not processed for histology were transferred to polypropylene tubes (Sarstedt Ltd., Leicester, England), snap-frozen in liquid nitrogen,

and stored at  $-70^{\circ}\text{C}$  until processed. A 5 ml blood sample was taken into ethylenediamine tetraacetic acid (EDTA) from each patient on admission.

### Polymerase chain reaction (PCR)

#### Specimen processing

Preparation of carotid arteries DNA was extracted from 25–30 mg of the carotid tissue from each patient using the QIAamp Tissue Kit (Qiagen Ltd., Crawley, England). The extract was stored at  $-70^{\circ}\text{C}$  until tested.

Preparation of White Cells And Plasma. Whole blood in EDTA was spun in a plastic conical centrifuge tube (Nunc, Kamstrup, Denmark) at 330 g for 5 minutes and the plasma removed to a 1.8 ml polypropylene tube (Sarstedt Ltd., Leicester, England). The cell pellet was made up to 10 ml with red cell lysing buffer containing 0.15 M  $\text{NH}_4\text{Cl}$ , 0.01 M  $\text{NaHCO}_3$  and 1 mM EDTA (Sigma, Poole, England), left at room temperature for 15 minutes and spun at 330 g for 5 minutes. The supernatant was replaced with 10 ml of PBS and the tube inverted gently 4 times. After a second spin at 330 g for 5 minutes the supernatant was removed and the cells counted in a Kova Glasstic 10 well grid (Hycor Biomedical Inc., California, USA) before making up to a final concentration of  $1 \times 10^7$  cells/ml in white cell lysing buffer constituted from 4 M guanidinium isothiocyanate in 3 M sodium acetate (Sigma, Poole, England) at pH 6.0. A volume of 200  $\mu\text{l}$  (equivalent to  $2 - 10^6$  cells) was extracted using the QIAamp Blood Kit ((Qiagen Ltd., Crawley, England). White cells and plasma were stored at  $-70^{\circ}\text{C}$  until tested.

#### Primers

The primers used are shown together with the size of their respective 1<sup>st</sup> and 2<sup>nd</sup> round products (Table 1).

### Mastermixes for PCR

The final 25  $\mu\text{l}$  volume of the the 1<sup>st</sup> round reaction mix for *Chlamydia* spp. nPCR contained 5 pmol and 25 pmol of the outer primers CHL2A and CHL2B respectively, 10 mM Tris-HCl pH 9.0, 3.5 mM  $\text{MgCl}_2$ , 50 mM KCl, 0.2 mM for each dNTP, 0.1% Triton X-100 and 0.625 units of Taq enzyme. The final 25  $\mu\text{l}$  volume for HLADRB PCR mastermix contained 5 pmol of each primer.

The final 25  $\mu\text{l}$  volume of the 2<sup>nd</sup> round reaction mix for *Chlamydia* spp contained 10 pmol and 25 pmol of the inner primers CHL2C and CHL2D respectively, 10 mM Tris-HCl pH 9.0, 3.5 mM  $\text{MgCl}_2$ , 50 mM KCl, 0.2 mM for each dNTP, 0.1% Triton X-100 and 0.625 units of Taq enzyme.

Extracted specimens (5  $\mu\text{l}$ ) were added to 20  $\mu\text{l}$  of the first round mix and subjected to the 1<sup>st</sup> round cycling

**Table 1: Primers for *Chlamydia* spp. (CHL2A-2D) and HLADRB gene (Amp A, Amp B).**

Primer	Sequence	Specificity
CHL2A	5'-GAA AAA ACT CTT RAA RTC GG-3'	MOMP gene 180/183 bp
CHL2B	5'-CGN ANG CTW ATR GCR TCR CAC CAA G-3'	MOMP gene 180/183 bp
CHL2C	5'-TGC CTG TRG GGA AYC CWK CTG AWC CAA G-3'	MOMP gene 94 bp
CHL2D	5'-CAA GTN CNR CAA GGA TCR CAA GGA TC-3'	MOMP gene 94 bp
AMPA	5'-CCC CAC AGC ACG TTT CYT G-3'	HLADRB gene 274 bp
AMPB	5'-CCG CTG CAC TGT GAA GCT CT-3'	I HLADRB gene 274 bp

Primers A&B were outer primers and C&D were inner primers.

conditions. A 0.5 µl volume of the reaction product from the first round was transferred to 24.5 µl of second round mix for the 2nd round.

#### Cycling conditions

Hot-start PCR was carried out in a Perkin Elmer GeneAmp 2400 thermal cycler. Cycling conditions are given in Tables 2,3.

**Table 2: PCR Cycling Conditions: Chlamydia genus specific**

Cycle Conditions	First Round	Second Round
PrePCRdenaturation	94°C – 3 min	94°C – 2 min
Denaturation	94°C – 10 s	94°C – 10 s
Annealing	45°C – 10 s	45°C – 10 s
Extension	72°C – 30 s	72°C – 30 s
Number of Cycles	35	35

First and second round products were visualised together on ethidium bromide-stained 2% agarose gels and photographed (Polaroid). Specimens were regarded as positive if an appropriately sized band was present on 1<sup>st</sup> and/or 2<sup>nd</sup> round PCR. *C. trachomatis*, *C. pneumoniae* and NFW controls were included in each round for the respective PCR.

**Table 3: PCR Cycling Conditions: HLA DRB gene**

Cycle Conditions	First Round
PrePCRdenaturation	96°C – 5 min
Denaturation	96°C – 1 min
Annealing	55 °C – 1 min
Extension	72°C – 1 min
Number of Cycles	40

#### Assay Sensitivity

Stocks of *C. trachomatis* and *C. pneumoniae* of known TCID<sub>50</sub> were used to determine the detection endpoints for the first and second round of the generic nPCR. The 1<sup>st</sup> and 2<sup>nd</sup> round endpoints were 10<sup>-4</sup> and 10<sup>-7</sup> for *C. trachomatis* and 10<sup>-3</sup> and 10<sup>-6</sup> for *C. pneumoniae* equating to 1 and 0.001 TCID<sub>50</sub> respectively.

#### Determination of TCID<sub>50</sub> for *C. trachomatis* and *C. pneumoniae*

Shell vials were seeded with McCoy cells grown in McCoy growth medium (MEM Eagles, inactivated fetal calf serum, glutamine, non essential amino acids, gentamicin) and incubated for 24 hrs at 37°C in a 10% CO<sub>2</sub> environment. Logarithmic dilutions of 500 µl of *C. trachomatis* cell culture controls from 10<sup>-3</sup> to 10<sup>-7</sup> were added to the shell vials in rows of 4 for each dilution. After centrifugation at 2000 rpm for 1 hour at room temperature the vials were incubated for 48 hours at 34°C in a 10% CO<sub>2</sub> environment. After 48 hours each shell vial was washed with PBS and fixed in methanol. 150 µl of a 1:5 dilution of anti-chlamydia direct immunofluorescence reagent (Dako) was added to each shell vial and incubated for 1 hour at 37°C. Following a further wash with PBS the vials were examined for elementary bodies under the immunofluorescence microscope. *C. pneumoniae* was grown in Hep2 cells using the same protocol and growth medium as described above. Logarithmic dilutions of 500 µl of *C. pneumoniae* cell culture controls from 10<sup>-2</sup> to 10<sup>-5</sup> were added to the shell vials in rows of 4 for each dilution. For both organisms 1 TCID<sub>50</sub> was calculated using the Karber formula.

#### PCR of specimens

All carotid specimens and white cells were tested using generic chlamydia nPCR. Aliquots of extracted DNA from all carotid specimens were spiked with *C. trachomatis* DNA at a weak dilution of 10<sup>-6</sup> (0.01 TCID<sub>50</sub>) and retested using the generic chlamydia nPCR to monitor for PCR inhibition. PCR for HLADRB gene was amplified in all carotid specimens to ensure adequate DNA was ex-

tracted. Only carotid specimens from which the HLADRB gene was amplified were included in the study and tested with chlamydia nPCR.

#### Serology

The MRL Diagnostics Chlamydia MIF (micro-immunofluorescence) assay [10] was used to detect the presence of IgG antibodies to antigens from *C. pneumoniae* (TW183), *C. psittaci* (strains 6BC and DD34) and *C. trachomatis* (sero-types D-K) at a serum dilution of 1/16. Slides were viewed at a magnification of 400 on a Leitz fluorescence microscope within 24 hours by two independent observers. Results were graded on a rank scale 0, 1+, 2+, 3+ and 4+.

#### Histology

Carotid specimens fixed in 10% formalin and embedded in paraffin wax were sectioned and stained with Haematoxylin & Eosin. Specimens were graded histologically as follows on microscopic examination by 2 investigators: Grade 0 normal artery, Grade 1 intimal thickening, smooth muscle cell damage, macrophage and foam cell infiltration, Grade 2 central necrotic area with overlying fibrosis, cholesterol clefts and Grade 3 dense fibrosis, calcification, ulceration, neovascularisation or haemorrhage [9]

## Results

### Patients and Specimens

Three of the 44 cases and their paired controls were excluded when the HLA DRB gene failed to be amplified from the atheromatous carotid specimens. Patient characteristics are summarized in Table 4.

**Table 4: Characteristics of patients with carotid artery disease that underwent carotid endarterectomy and were enrolled in the study.**

Characteristic	No.(%) of patients
Men	25 (66)
Women	13 (34)
Hypercholesterolaemia	21 (55)
Hypertension	11 (29)
Diabetes mellitus	1 (3)
Family history of CVA <sup>*</sup> /MI <sup>φ</sup> /PVD <sup>#</sup>	16 (42)
Smoker	6 (16)
Ex-smoker	20 (53)
Previous CVA/MI	19 (50)

\*CVA = cerebrovascular accident, <sup>φ</sup>MI = myocardial infarction, <sup>#</sup>PVD = peripheral vascular disease

### PCR of specimens

*Chlamydia* spp. were not detected in any carotid atheroma specimens. All the white cell specimens were negative for *Chlamydia* spp. Inhibition of the generic nPCR was not demonstrated in any carotid specimen when each aliquot was spiked with target *C. trachomatis* at a weak dilution of 10<sup>-6</sup>.

### Serology

Twenty-five out of 38 (66%) plasma specimens were positive for *C. pneumoniae* IgG. Two patients who had antibodies to *C. pneumoniae* were also positive for *C. trachomatis* IgG and one for *C. psittaci* IgG.

### Histology

Apart from the first three carotid specimens from patients and four post mortem control specimens, all specimens were fixed in formalin for retrospective confirmatory histological grading following macroscopic grading. Of the 41 control specimens that were grade 0 macroscopically, 11/41 (27%) were grade 1 and 2/41 (5%) were grade 2 on histological examination. Thirty disease specimens were grade 3 macroscopically of which 26 were also grade 3 and 1 was grade 2 on histology. The first 3 specimens were not examined histologically. Nine of 10 specimens of grade 2 on gross examination were confirmed as grade 2 and 1 as grade 1 on histology. One specimen was grade 1 on both macroscopic and microscopic examination.

### Discussion

The number of specimens analyzed in this study was relatively small therefore the null finding could be due to chance. Previous meta-analysis showed the occurrence of *C. pneumoniae* in atheromatous lesions compared to non-atheromatous vessels of controls was 59% versus 3.1%[8]. In our study *Chlamydia* spp. were not detected in any of the atheromatous carotid specimens which is the same as detection rates in a study of abdominal aortic aneurysms carried out by Lindholt *et al*[11]. Weiss *et al* only detected *C. pneumoniae* in one coronary atheroma [6]. *C. pneumoniae* was only detected in 4/50 (8%) coronary atherectomies from patients in Germany [7].

Several measures were taken to ensure that our results were not false negatives. The sensitivity of the nPCR was determined by comparing the endpoints with titrations of *C. trachomatis* and *C. pneumoniae* of known TCID<sub>50</sub>. The nested approach gave a 10<sup>3</sup> increase in sensitivity. This was to ensure that the lack of detection of *Chlamydia* spp. was not due to lack of sensitivity of the nPCR. We looked for the presence of inhibitors in the tissue specimens. An aliquot of each extracted DNA specimen was spiked with a weak dilution of *C. trachomatis* DNA and amplified by PCR to exclude such inhibitors. We did

not demonstrate any inhibition to our assay. In retrospect it would have been useful to spike the tissue sample with whole chlamydia particles prior to extraction to determine whether chlamydia DNA was being lost at the extraction step. Further studies should look at this aspect.

The size of specimens and method of DNA tissue extraction may account for the differences in the rate of detection. The heterogeneity of distribution of the organism within the tissue may elude detection by various methods as noted by Jackson et al [4]. Jantos et al [7] who used a different method and Paterson et al [12] who looked at larger samples of carotid endarterectomy and carotid and coronary arteries from autopsy also failed to detect *C. pneumoniae*. In this study the Qiagen tissue DNA kit, which is specific for DNA extraction from tissue samples, was used. The cumulative tissue analyzed in this study argues against the negative findings resulting from inadequate amounts of tissue investigated. In order to ensure that adequate DNA was extracted in this study, the HLADR gene was amplified in each specimen. Results of chlamydia PCR in each individual specimen were only considered valid when the HLADR gene was successfully amplified and when inhibition to the PCR was excluded. Care was also taken at all stages to avoid contamination of specimens, especially at the stage of DNA tissue extraction.

The difficulty of obtaining adequate numbers of disease-free and age and sex matched controls has been a problem in previous investigations, especially in an elderly population. Taylor-Robinson suggested the best control comprises disease-free specimens adjacent to abnormal areas in vessels from the same subjects [8]. One study showed that *C. pneumoniae* was not detected by electron microscopy in adjacent tissue without disease [5]. In our study external controls from 8 age and sex matched post mortem patients were used when we were not able to identify adjacent tissue free of atheroma on macroscopic examination in patient specimens. Although 13/41 (32%) of the controls were found retrospectively on histology to have signs of early atheroma development, *Chlamydia* spp. were not detected. There is much evidence from many workers that *C. pneumoniae* is present in atheromatous material including studies using electron microscopy, antigen detection and even culture.[13] However sensitive techniques such as PCR should be the gold standard for detection of organisms at low levels in atheromatous material. The wide range in detection rates in the literature is therefore surprising.

Apfalter et al have recently published a multicenter comparison trial of DNA extraction methods and PCR assays for detection of *C. pneumoniae* in endarterectomy speci-

mens.[14] Despite this study an explanation for the wide discrepancies is unclear. In the Apfalter *et al.* study, the majority of methods (10/16) found all 15 specimens negative. Hence the majority consensus among the expert laboratories was that all the specimens were negative. Three methods were able to detect the low positive control (0.01 inclusions). Of these 3 most sensitive methods 2/3 found all 15 specimens negative. In contrast, of the 2 laboratories that detected *C. pneumoniae* in the most clinical specimens, one does not detect the lowest positive control. This less sensitive laboratory/method (A) was the one that had the most positive results (9/15).

Unfortunately the study design employed by Apfalter *et al.* was not ideal for assessing specificity as only one negative control was employed. Suspiciously, the two laboratories that detect the most positives share a common extraction methodology (Boehringer High Pure PCR template preparation kit). This is a relatively intricate method with 6 spin steps. The consequent increase in manipulation steps over that associated with other extraction methods makes this method inherently potentially prone to amplicon and carry-over contamination. Working in a routine clinical laboratory, we avoid such potentially non-robust methods because of the potential associated specificity problems. The positive control data (samples C1-4) do not suggest that the Boehringer High Pure PCR template preparation kit confers a higher sensitivity as 2 methods not using this kit had better sensitivity than method A, and a further 7 labs had equivalent sensitivity to method A. The two 'best' laboratories yield 9/15 (lab A) and 5/15 (lab B) positive results. The concordance between these two laboratories is not accessible from the data presented in the paper. We are not told if the 5/15 positives in lab B are included in the 9/15 positives in lab A. In addition the positive PCR products were not sequenced. Thus there was no attempt to show if the positive products represent real positive samples or are related to amplicon or carry-over contamination from laboratory or control samples. PCR contamination is an insidious problem. PCR contamination remains a viable explanation for the constellation of results presented by Apfalter *et al.* It is possible that the literature in this area is biased by false positive PCR results.

This work in the current study was carried out in a diagnostic laboratory (Regional Virus Laboratory, Belfast). We routinely use nested PCR assays as diagnostic procedures. In this setting we necessarily place much emphasis on specificity, including robust extraction methodologies and minimization of potential for contamination.

A publication bias against negative findings in well-conducted studies is something to be guarded against as it

compromises future systematic review. We feel that this may be a particular problem in this subject area. We feel that there is a particular role for electronic journals such as BMC in which space, in contrast to paper journals, is not a limitation. Unlimited publication space will increase the chance that papers with negative findings are published.

*Chlamydia* spp. were not detected in carotid atheroma in this group of patients despite a high prevalence of *C. pneumoniae* antibodies comparable to that previously reported in the Northern Ireland population [15], an area with a high prevalence of atherosclerotic disease. Weiss et al who only detected the organism in one coronary atheroma by PCR also found that seroprevalence for *C. pneumoniae* antibodies did not correlate with the presence of *C. pneumoniae* in coronary atheroma in the patients in his study [6]. This implies that if there is any causal association between *C. pneumoniae* and atherogenesis then it must be indirect or that it is a variable that is not essential for atheroma development.

## Conclusion

In this study we were not able to detect the presence of *Chlamydia* spp. in atheromatous specimens using nPCR. Hence we are not able to support the direct involvement of chlamydia in the atherogenesis of carotid disease. Even if *C. pneumoniae* is present in atheromatous material it does not indicate that there is a causal association of infection with the organism and atherosclerosis. Other factors such as alteration of lipid profile [16], cytokine mediated endothelial injury [17,18] molecular mimicry [19] or immune cross reactivity [20] may be an explanation for an indirect relationship of *C. pneumoniae* with atherogenesis considering the high prevalence of *C. pneumoniae* antibodies in this population.

## Competing interests

None declared.

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## Pre-publication history

The pre-publication history for this paper can be accessed here:

<http://www.biomedcentral.com/content/backmatter/1471-2334-1-12-b1.pdf>