Isolation of *Chlamydia pneumoniae* from a Carotid Endarterectomy Specimen


*Chlamydia pneumoniae* has been associated with atherosclerotic cardiovascular disease by both seroepidemiologic studies and direct detection of the organism in atherosclerotic plaque by electron microscopy (EM), immunocytochemistry (ICC), and polymerase chain reaction (PCR). Despite the frequent detection of the organism in atheromatous cardiovascular specimens by these methods, only 1 cardiovascular isolate of *C. pneumoniae*, obtained from a coronary artery, has been previously reported. This study reports the isolation of *C. pneumoniae* from a prospectively obtained carotid endarterectomy specimen. The organism appears to be identical to other *C. pneumoniae* isolates by EM morphology, reactivity to species-specific monoclonal antibodies, and Southern hybridization analysis of chromosomal digests using *C. pneumoniae*-specific DNA probes. *C. pneumoniae* was detected by PCR or ICC (or both) in 11 (69%) of 16 other endarterectomy specimens tested by both of these methods. These results provide further evidence for an association of *C. pneumoniae* and atherosclerosis by confirming the presence of viable bacteria within atherosclerotic plaque.

*Chlamydia pneumoniae* has been associated with atherosclerotic cardiovascular disease both by seroepidemiologic studies, indicating a significantly higher prevalence of circulating *C. pneumoniae* antibody or immune complexes among persons with clinical or radiographic evidence of atherosclerotic disease [1, 2], and by direct detection of the organism in atherosclerotic plaque. The organism has been detected by electron microscopy (EM), immunocytochemistry (ICC), direct immunofluorescence, and the polymerase chain reaction (PCR) in coronary artery [3–5], aortic [6], and carotid artery [7] plaque specimens.

These detection methods, while indicating that *C. pneumoniae* is present in a large proportion of atheroma specimens from multiple cardiovascular sites, do not allow further characterization of the organisms found in plaque.

Chlamydiae are slowly growing, obligate intracellular bacteria that have traditionally been difficult to isolate from clinical specimens. Although multiple investigators have attempted to isolate *C. pneumoniae* from atheroma specimens, to date only 1 such isolate has been reported, from the coronary artery of a transplant recipient’s excised heart [8]. Other studies have been limited, either by the extremely small amounts of tissue available for testing, as with atherecotomy samples, or by the need to freeze and store specimens for transport, which decreases the likelihood of isolation of the organism.

To overcome some of these difficulties, we initiated a prospective study of patients undergoing carotid endarterectomy, a procedure during which relatively large amounts of atheromatous material is removed. This allowed specimens to be transported to our laboratory for inoculation onto cell culture within hours of surgical removal. We report the suc-
cessful isolation of *C. pneumoniae* from a carotid atheroma specimen.

**Materials and Methods**

**Patients and specimens.** Specimens were obtained from patients undergoing routine and emergent carotid endarterectomy at two hospitals in the greater Seattle area. After surgical removal, specimens were immediately placed in sterile chlamydia transport (sucrose–phosphate–glutamic acid, SPG) medium and refrigerated. They were then transported to the laboratory on wet ice, usually within 6 h. On arrival at the laboratory, a portion of the specimen was ground, resuspended in SPG, and inoculated onto HL cells for isolation. Initially, the entire remaining portion of the specimen was resuspended in DNA-extraction buffer for PCR. Midway through the study the protocol was amended to include ICC testing of specimens, and the portion of the specimen not inoculated onto cell culture was then divided, with one part processed for PCR and one part fixed in formalin for ICC.

To minimize the risk of contamination, tissue homogenization, sample preparation, tissue culture, PCR amplification, and product analysis were done in separate rooms. The rooms used for tissue and for sample preparations were equipped with germicidal UV light bulbs. The PCR sample room is used for this purpose only.

**Isolation and characterization of isolates.** After a 3- to 6-day incubation period, the cell cultures were stained with a *C. pneumoniae*-specific fluorescent-conjugated monoclonal antibody and examined for intracellular inclusions using a fluorescent microscope. If an organism was identified, it was passed continuously in cell culture for characterization. It was then tested for similarity to prototype *C. pneumoniae* isolates by typing with *C. pneumoniae* species-specific monoclonal antibodies, examination of inclusion morphology in cell culture, examination of ultrastructural morphology by EM, Southern hybridization analysis of chromosomal digests using *C. pneumoniae*-specific DNA probes, and analysis of amplified major outer membrane protein (MOMP) gene sequences.

Southern hybridization analysis of chromosomal digests was done as previously described with minor modifications [9]. Briefly, five different *C. pneumoniae*-specific fragments were labeled with digoxigenin-dUTP according to the manufacturer’s directions (Boehringer Mannheim, Indianapolis). Following gel electrophoresis of DNA prepared from the isolate and *C. pneumoniae* AR-39, DNA digested with PstI or BamHI was transferred to 0.2 μm noncharged nylon membranes (Qiabrade; Qiagen, Chatsworth, CA). Hybrids were detected using the Genius nonradioactive detection system according to the manufacturer’s instructions (Boehringer Mannheim).

The variable domain region IV of the MOMP gene, which is highly variable among *Chlamydia trachomatis* isolates, was amplified using the Cpn 201/202 primer pair as described by Gaydos et al. [10]. Subsequently, the entire MOMP gene was sequenced using primers derived from published *C. pneumoniae* MOMP sequences, which generated three overlapping products of 587, 482, and 569 bp, spanning the entire gene. Amplified product was gel-purified using a kit (BandPrep, Pharmacia, Piscataway, NJ; or gel extraction, Qiagen) according to the manufacturer’s directions. Subsequently, PCR products were directly sequenced (Ready Reaction kit, Applied Biosystems, Foster City, CA; or ThermoCycle kit, Perkin-Elmer Cetus, Norwalk, CT). Unincorporated ddNTPs were removed (SpinColumn; Pharmacia) and sequencing was accomplished (377 sequencer; Applied Biosystems).

**ICC.** ICC staining with a *Chlamydia* genus-specific mouse monoclonal antibody (CF-2) was performed on adjacent 5-μm sections of the formalin-fixed specimen, using methods previously described [7].

**PCR.** PCR was done using the HL-1 and HR-1 primer sets as previously described [11]. DNA was purified with a boiling method. Briefly, sections were boiled in a 5% suspension of chelating resin (Chelex 100; Sigma, St. Louis) in sterile water. The resulting supernatants were extracted with phenol-chloroform by standard methods and precipitated with ethanol, and the DNA pellets were resuspended in 50 μL of TRIS-EDTA buffer at pH 8. Mock extractions of buffer were done and amplified to ensure that no contamination occurred. PCR reagents without any specimen and various dilutions of purified *C. pneumoniae* DNA were included in each PCR run as the negative and positive controls, respectively. Confirmation of presumptive positives and detection of products below the sensitivity of agarose gels was done by immunochemiluminescence as described [4]. If inhibition of PCR was observed, drop dialysis against sterile water was performed and PCR was repeated.

**Serology.** Serology was done using the microimmunofluorescence (MIF) test as previously described [12].

**Results**

Endarterectomy specimens obtained from 25 patients were inoculated onto cell culture. Of those, *C. pneumoniae* was isolated from 1 specimen, from a 60-year-old woman who underwent elective carotid endarterectomy. The organism was isolated from cell culture after a 6-day incubation period and was typed as *C. pneumoniae* by reactivity with *C. pneumoniae*-specific monoclonal antibodies. Three isolates per coverslip were recovered in the first passage; 68 isolations per coverslip were recovered in the second. EM demonstrated pear-shaped elementary bodies with morphology typical of *C. pneumoniae* (figure 1). The isolate was identical to prototype isolates of *C. pneumoniae* by Southern hybridization analysis of chromosomal digests using *C. pneumoniae*-specific DNA probes, indicating identical hybridization patterns, and sequence analysis of amplified MOMP gene sequences, showing complete homology. *C. pneumoniae* was not detected in other portions of the plaque specimen tested by ICC or PCR.

A serum specimen obtained from the patient 1 month after surgery had an anti–*C. pneumoniae* IgG antibody titer of 128 by MIF. IgM and IgA anti–*C. pneumoniae* antibodies were not detected.

Specimens from all patients were tested by PCR, and specimens from 16 of the 25 were also tested by ICC. The organism was detected by PCR in 6 (24%) of 25 and by ICC in 8 (50%) of 16 specimens tested. *C. pneumoniae* was identified by PCR, ICC, or isolation in 12 (75%) of 16 specimens tested by all of these methods. Both PCR and ICC were positive in 3 of these 16 specimens.
Discussion

These results provide further evidence for an association of C. pneumoniae and atherosclerosis by confirming the presence of viable organisms within atherosclerotic plaque. The results of the DNA hybridization and MOMP gene sequence analysis indicated no differences between the carotid plaque isolate and prototype C. pneumoniae respiratory strains. This suggests that the organisms detected in atheroma are likely to be similar to those isolated in conjunction with acute respiratory infections.

The results of serology for the culture-positive patient are compatible with previously defined criteria for prior infection with C. pneumoniae (IgG ≥16 and ≤512). These findings are consistent with those of a recently reported study, in which 23 of 26 patients with C. pneumoniae detected by PCR in surgical aortic aneurysm specimens had serologic results consistent with past infection with C. pneumoniae [6]. Not all patients with C. pneumoniae organisms in atheroma have met this serologic criteria, however. C. pneumoniae has been detected by PCR or ICC in coronary atherectomy and aortic aneurysmal specimens from persons seronegative for C. pneumoniae [4, 6] and in carotid plaque specimens from patients with IgG titers ≤16 [7]. Since anti-C. pneumoniae IgG titers decline following acute infection [13], it is likely that the low or undetectable titers found in some patients with PCR- or ICC-positive atheroma specimens represent a decline from levels that occurred in response to the previous acute C. pneumoniae infection.

The organism was not detected by PCR or ICC in other portions of the culture-positive endarterectomy specimen. This may be due to heterogeneity in the distribution of the organism within plaque specimens. We have noted discordant results for adjacent sections of atherectomy specimens tested by PCR and ICC [4] and differential detection of the organism by ICC when multiple fragments of a single carotid atheroma obtained during endarterectomy were tested [7]. False-negative results by PCR are also possible due to tissue inhibitors which may be present in clinical samples. Contamination of the specimen is a possible but unlikely explanation for the isolation, since rigorous procedures were followed to minimize the risk of contamination from other sources. In addition, since the specimen was coincidentally received on the day before the 4-day Thanksgiving holiday, no other specimens were processed in the laboratory that day.

PCR and ICC testing of the other 24 carotid endarterectomy specimens indicates that the organism is present in a large proportion of atheroma specimens. The organism was detected by either PCR or ICC in 11 (69%) of 16 specimens tested by both methods. These results are compatible with our previously reported findings with other carotid specimens, in which the organism was detected by ICC in 59% (36/61) of prospectively obtained and archival endarterectomy samples [7].

The results of this and previous studies indicate that, while C. pneumoniae may be isolated from atheroma, this should not be the primary method used in studies evaluating the prevalence of C. pneumoniae in such specimens. The rarity of isolation compared with the high rates of detection of C. pneumoniae antigen and DNA by ICC and PCR, respectively, is consistent with the results of studies of other chronic chlamydial infections, specifically, trachoma and pelvic inflammatory disease due to C. trachomatis.

In those infections, C. trachomatis DNA or antigen may be demonstrated in a high proportion of affected tissues while
the organism is rarely recovered [14, 15], suggesting that the organism persists in an uncultivable state. These findings may in part be explained by the presence of relatively low numbers of chlamydial elementary bodies, the infectious form of the organism, in chronically infected tissues.

Isolation of the organism from carotid atheroma in the present study provides important information concerning the viability and characteristics of C. pneumoniae found in plaque specimens. As with other studies reporting detection of the organism in atheromatous material, these results do not establish a causal role for the organism in the development or propagation of atheromatous lesions. These findings should be pursued with additional animal model studies and human intervention trials, which are better able to address this hypothesis.

Acknowledgments

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References