Distribution of *Chlamydia pneumoniae* DNA in Atherosclerotic Carotid Arteries: Significance for Sampling Procedures

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Despite extensive efforts to confirm a direct association between *Chlamydia pneumoniae* and atherosclerosis, different laboratories continue to report a large variability in detection rates. In this study, we analyzed multiple sections from atherosclerotic carotid arteries from 10 endarterectomy patients to determine the location of *C. pneumoniae* DNA and the number of sections of the plaque required for analysis to obtain a 95% confidence of detecting the bacterium. A sensitive nested PCR assay detected *C. pneumoniae* DNA in all patients at one or more locations within the plaque. On average, 42% (ranging from 5 to 91%) of the sections from any single patient had *C. pneumoniae* DNA present. A patchy distribution of *C. pneumoniae* in the atherosclerotic lesions was observed, with no area of the carotid having significantly more *C. pneumoniae* DNA present. If a single random 30-μm-thick section was tested, there was only a 35.6 to 41.6% (95% confidence interval) chance of detecting *C. pneumoniae* DNA in a patient with carotid artery disease. A minimum of 15 sections would therefore be required to obtain a 95% chance of detecting all true positives. The low concentration and patchy distribution of *C. pneumoniae* DNA in atherosclerotic plaque appear to be among the reasons for inconsistency between laboratories in the results reported.

*MATERIALS AND METHODS*

**Patients and serology.** This study was conducted with the approval of the Queensland University of Technology Human Ethics Committee (1736H). The study analyzed a subset of 10 patients from a larger cohort of 54 CAD patients who were undergoing elective surgery (carotid endarterectomy). All patients gave their informed consent prior to surgery. Atherosclerotic plaques removed at the time of surgery were immediately fixed in 10% formaldehyde for subsequent PCR analysis. The presence of serum antibodies to *Chlamydia* was determined by the MRL Diagnostics (Cypress, Calif.) immunoglobulin G (IgG) microimmunofluorescence (MIF) test and the Medac Diagnostika (Wedel, Germany) IgG-IgA recombinant enzyme-linked immunosorbent assay (ELISA). The serum samples were considered positive when there were titers of ≥32 for the MIF assay and ≥100 for the Medac assay.

**Preparation of carotid arteries for PCR.** Each carotid artery was cut transversely into smaller sections of ~5 mm, and their positions in relation to the carotid bifurcation were recorded. Each portion of formalin-fixed plaque specimen was decalcified, embedded in paraffin, and processed according to conventional techniques. A series of 5-μm-thick sections were cut. Six sequential sections (total sample, 30 μm) were pooled and assayed by a *C. pneumoniae*-specific nested PCR. At least five serial sets of 30 μm for PCR followed by 15 μm for histopathology were cut from four highly stenotic areas adjacent to the carotid bifurcation. For each patient, therefore, this involved the testing of a minimum total of 20 sets of 30 μm of plaque by PCR.

DNA was extracted from each pooled 30-μm section of paraffin-embedded carotid tissue from each patient using the QIAamp tissue kit (Qiagen, Melbourne, Australia). The 60 μl of DNA extract was stored at −70°C until it was tested.

**ompA PCR.** *C. pneumoniae* DNA was detected using a nested PCR assay targeting a 366-bp fragment of the *C. pneumoniae* *ompA* gene (1). The nested PCR used was highly specific and could detect as few as 10 chlamydial bodies. For both rounds, DNA (minimum concentration, 10 ng per PCR) was amplified in 25-μl volumes containing 1 μM primers, 200 μM deoxyribonucleotides, 1× PCR buffer, 1.2 U of Taq polymerase, and 1 μl of the DNA sample or first-round template. Each sample was tested in conjunction with controls: negative controls with no template added and positive controls containing 100 copies of *C. pneumoniae* 446-bp first-round PCR product. Negative controls were included after every third test sample to ensure that there was no aerosol contamination. Ten percent of the test samples were spiked with 100 copies of *C. pneumoniae* 446-bp first-round PCR product to test for PCR inhibition. Each sample was tested in duplicate. At least one of the two duplicates needed to be PCR positive for the

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**Chlamydia pneumoniae** is a gram-negative obligate intracellular bacterium that is responsible for 10% of community-acquired pneumonia (3). Infection with *C. pneumoniae* appears to be geographically widespread, with seroepidemiological studies showing that 40 to 70% of adults have been infected at least once in their lifetime (10). In addition to the bacterium’s role in respiratory disease, there is growing evidence that *C. pneumoniae* may be involved in the pathogenesis of atherosclerosis. Seroepidemiological studies first implicated *C. pneumoniae* as an independent risk factor for cardiovascular diseases (12). Since then, *C. pneumoniae* has been detected in human atherosclerotic lesions by various techniques, including cell culture (4), electron microscopy (7), immunohistochemistry, and PCR (4, 5). While detection rates vary considerably, *C. pneumoniae* has been detected, on average, in 59% of atheromatous arteries and rarely in non diseased arteries (15).

Despite extensive efforts to confirm a direct association between the bacterium and atherosclerosis, different laboratories continue to report a large variability (from 0 to 100%) in detection rates (5, 9). These large variations may be attributed to either (i) differences in detection methodology or (ii) the irregular distribution of *C. pneumoniae* in the plaque, influencing sample selection and hence positivity rates. The aim of this study was to determine the location and number of sections of the plaque that are required for analysis to obtain a 95% confidence interval (CI) for detecting the bacterium in patients with carotid artery disease (CAD).
specimen to be recorded as \textit{C. pneumoniae} positive. All duplicates were randomized and tested on different days, and the results were scored in a blinded manner. DNA sequencing was performed on selected PCR products to confirm that \textit{C. pneumoniae} DNA was indeed being amplified. Sequencing of the PCR product was done using the standard dideoxy sequencing method of Sanger et al. (13). All samples were confirmed as \textit{C. pneumoniae} using a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST), and ecrustalw in WebANGIS was used to align the existing \textit{C. pneumoniae} sequences to the test samples. All sequences were 99.7% homologous with previously documented strains of \textit{C. pneumoniae}.

\textbf{Statistical analysis.} Resampling techniques were employed to determine the likelihood of successfully detecting \textit{C. pneumoniae} in 1 to 20 sections from a CAD patient. In order to avoid making distributional assumptions from the resampling results, the 95% CIs were generated from the percentiles 2.5 and 97.5%.

\section*{RESULTS}

All patients displayed at least two of the established risk factors for cardiovascular disease (age, sex, hyperlipidemia, hypertension, hyperhomocysteinemia, ischemic heart disease, diabetes, family history of premature cardiovascular disease, and smoking status). Serum specimens from the nine patients (excluding patient 8) were examined by MIF tests and ELISA for the presence of antibodies to \textit{C. pneumoniae}. Antibody titers from three of the nine patients (patients 5, 6, and 7) indicated a preexisting infection with \textit{Chlamydia} by the MIF test. The ELISA indicated that two of the nine patients (patients 3 and 7) had acute \textit{C. pneumoniae} infections. The MIF and ELISA results did not correlate for three of the seropositive patients.

\textit{C. pneumoniae} was detected in all 10 of the atherosclerotic carotids (Table 1). However, the percentage of sections that were PCR positive and the reliability of the duplicates varied considerably, both between patients and also at different sites within the plaque. On average, 42\% (ranging from 5 to 91\%) of the sections from any single patient had \textit{C. pneumoniae} DNA present. PCR inhibition was not a significant problem, since when 10\% of the samples were spiked with 100 molecules of \textit{C. pneumoniae} DNA, no PCR inhibition was observed. Of >20 PCR products that were extracted from the gels and sequenced, all were confirmed as true \textit{C. pneumoniae} products.

Two criteria were used to determine if a section of plaque was \textit{C. pneumoniae} positive: (i) either duplicate test should be positive (single positive) or (b) both duplicate tests must be positive (double positive). All patients were PCR positive for \textit{C. pneumoniae} using the single-positive criterion, while only 8 of the 10 patients displayed double positives (Table 1). The number of positive sections and the patchy distribution of \textit{C. pneumoniae} DNA were consistent in different regions of the diseased arteries (internal carotid and common carotid).

Resampling techniques were used to determine the chance of detecting \textit{C. pneumoniae} by PCR in 1 to 20 sections from the

\begin{table}[h]
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\small
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{Patient} & \multicolumn{4}{c|}{\textbf{No. of PCR-positive sections}} & \multicolumn{4}{c|}{\textbf{Double positive}} \\
 & \textbf{Internal carotid} & \textbf{Bifurcation} & \textbf{Common carotid} & \textbf{Total % PCR positive} & \textbf{Internal carotid} & \textbf{Bifurcation} & \textbf{Common carotid} & \textbf{Total % PCR positive} \\
\hline
1 & 5/5 & 16/7 & 7/7 & 4/5 & 91 & 5/5 & 10/17 & 2/7 & 2/5 & 54 \\
2 & 5/5 & 3/5 & 0/5 & 3/5 & 55 & 5/5 & 3/5 & 0/5 & 3/5 & 55 \\
3 & 2/6 & 1/5 & 13/22 & 5/12 & 47 & 0/6 & 0/5 & 6/22 & 1/12 & 16 \\
4 & 3/5 & 5/5 & 1/5 & 0/5 & 45 & 1/5 & 1/5 & 0/5 & 2/5 & 20 \\
5 & 2/5 & 1/5 & 1/5 & 5/5 & 45 & 0/5 & 0/5 & 0/5 & 0/5 & 0 \\
6 & 3/5 & 0/5 & 1/5 & 2/5 & 30 & 0/5 & 0/5 & 0/5 & 0/5 & 0 \\
7 & 0/5 & 1/5 & 1/5 & 4/5 & 30 & 0/5 & 1/5 & 3/5 & 0/5 & 20 \\
8 & 0/5 & 1/5 & 5/5 & 0/5 & 30 & 0/5 & 0/5 & 0/5 & 1/5 & 16 \\
9 & 0/5 & 0/5 & 0/5 & 1/5 & 5 & 0/5 & 0/5 & 0/5 & 1/5 & 5 \\
10 & 0/5 & 1/5 & 0/6 & 0/5 & 5 & 0/5 & 0/5 & 0/6 & 0/5 & 0 \\
\hline
\textbf{Average % positive} & 39 & 35 & 34 & 42 & 24 & 24 & 14 & 25 \\
\hline
\end{tabular}
\caption{Distribution of \textit{C. pneumoniae} DNA within atherosclerotic carotid arteries from 10 endartectomy patients$^a$}
\footnotesize{$^a$ Thirty-micrometer-thick sections from four 5-mm segments (internal carotid, bifurcation, and two adjacent segments [i and ii] of the common carotid) were analyzed per patient. Each section was tested in duplicate for \textit{C. pneumoniae}. Single positive, detected in at least one of the duplicates; double positive, detected in both duplicates. The fractions represent the number of PCR-positive sections out of the total number of sections tested.}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Success rate for detecting \textit{C. pneumoniae} by PCR in 1 to 20 sections from atherosclerotic carotid artery plaques. Each section was tested in duplicate. The error bars indicate the 95\% CI.}
\end{figure}
sample population (Fig. 1). The results showed that when a single random 30-μm-thick section was tested, there was only a 35.6 to 41.6% (95% CI) chance of detecting C. pneumoniae DNA in a patient. A minimum of 15 sections was required to obtain a 95% chance of detecting all true positives using the single-PCR-positive criterion. When only the test results that were repeatedly positive by PCR were included, the chance of detecting C. pneumoniae in one section was 22%.

DISCUSSION

When extensive sampling of the 10 patients was performed (a total of ≥20 30-μm-thick sections analyzed per patient), all 10 were found to have C. pneumoniae DNA present at one or more locations within the plaque. By comparison, if the present commonly used approach of testing a single section was used, then there was only a 35.6 to 41.6% (95% CI) chance of detecting C. pneumoniae DNA. The small amounts and patchy distribution of C. pneumoniae DNA in atherosclerotic plaque are among the reasons for the inconsistency between laboratories in results reported.

Based on the present patient group, 15 sections would need to be tested to have a 95% confidence level for recording true C. pneumoniae positives. Previous studies examining the presence of C. pneumoniae in human atherosclerotic arteries have usually tested a single 4- to 8-μm-thick section, while some have attempted to test the whole lesion (5, 16). When large amounts of plaque are tested, PCR inhibitory factors become problematic. By comparison, while inhibitors may be less of a problem with a single section, this approach will result in a gross underestimation of the true prevalence of the microorganism in patients with cardiovascular disease.

There was no obvious correlation between the presence of C. pneumoniae DNA and (i) the presence of C. pneumoniae antibodies in each patient or (ii) traditional cardiovascular disease risk factors. The lack of a direct relationship between C. pneumoniae DNA and antibodies was not unexpected, as this finding has been reported previously (2). Thus, serological tests do not appear suitable for predicting a vascular C. pneumoniae infection.

Nested PCR assay is considered a sensitive means of detecting C. pneumoniae DNA in atherosclerotic tissues, yet it is often problematic due to an increased risk of contamination. Processing of clinical specimens, preparation of the PCR mixture, and addition of template were all conducted in separate areas to avoid cross-contamination. The use of negative controls every third sample was included to ensure that no false positives were obtained.

Our findings have shown that replicate testing of each sample increased the proportion of PCR-positive specimens. We consider the results for patients for whom only one of the two duplicates was positive to be true positives and not due to contamination, as all negative controls were as expected. Previous publications have also reported inconsistencies with repeated PCR testing of samples (8, 11, 14, 17). Mahony et al. compared five different PCR assays and found that the sensitivity for detecting C. pneumoniae DNA from culture material was 0.005 inclusion-forming units/test, while the reproducibility decreased notably below 0.01 inclusion-forming units (8). Smieja et al. observed that repeat testing of the same sample increased the proportion of positive clinical specimens (14). This poor reproducibility may be attributed to the small amount of C. pneumoniae DNA present in the diseased artery, to sampling errors arising from the use of such small sample volumes, to the observation that chlamydiae tend to cluster, and to PCR inhibition. Thus, without extensive testing of the sample, any result will be an underestimation of the true prevalence of C. pneumoniae.

The quantity (on average, 42% of sections; 102 of 241 sections from 10 patients) and irregular distribution of C. pneumoniae DNA in the plaque were consistent findings that we observed throughout the diseased carotid artery. Only one other study has analyzed multiple sections from the same tissue specimen. Kuo and Campbell analyzed 167 sections from 51 positive patients and found 68% of the sections positive for C. pneumoniae by immunocytochemistry (6). A possible explanation for the slightly higher detection rate compared to our study may be due to methodological differences or to sampling various arterial tissues (aorta, carotid, and coronary arteries) rather than one arterial site (carotid). The aorta, coronary, and iliac arteries appear to have two to four times more C. pneumoniae DNA present than the carotid artery (16). Our study is the first to comprehensively examine the presence of C. pneumoniae throughout more than three sections of the carotid artery by PCR. Due to the low concentration and patchy distribution of C. pneumoniae DNA in atherosclerotic plaques, sampling procedures should analyze more sections from one arterial site to gain greater confidence in recording true positives. An improved understanding of the distribution of C. pneumoniae in atherosclerotic plaque provides important new information about the potential role of this organism in atherosclerosis.

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REFERENCES


