No Evidence of Involvement of *Chlamydia pneumoniae* in Severe Cerebrovascular Atherosclerosis by Means of Quantitative Real-Time Polymerase Chain Reaction

Petra Apfalter, MD; Wolfgang Barousch, BSc; Marion Nehr; Birgit Willinger, MD; Manfred Rotter, MD; Alexander M. Hirschl, MD

**Background and Purpose**—All studies reporting high numbers of *Chlamydia pneumoniae* DNA positives in stroke patients published to date have used polymerase chain reaction (PCR) techniques highly prone to generate false-positive results. The aim of this study was to analyze the prevalence of *C. pneumoniae* DNA in plaques of the carotid artery as well as in peripheral blood by means of a new, closed, real-time PCR system.

**Methods**—Carotid endarterectomy specimens and preoperative peripheral blood mononuclear cells (PBMC) of 75 individuals with severe cerebrovascular atherosclerosis were analyzed by means of a *C. pneumoniae*-specific quantitative ompA-based real-time PCR TaqMan system. Plaques were also cultured onto HEp-2 cells. Before the surgical intervention, *C. pneumoniae*-specific IgM, IgG, and IgA as well as C-reactive protein (CRP) levels were determined.

**Results**—89% of all patients studied had *C. pneumoniae*-specific antibodies, but the pathogen was not detected in a single carotid atheroma by real-time PCR and cell culture. However, *C. pneumoniae* DNA was detected in 4 PBMC samples (5.3%) at very low levels (≤1 inclusion/6 mL EDTA blood). No statistical significance was found between symptomatic/asymptomatic patients, *C. pneumoniae* PCR, results and CRP values after correction for multiplicity-of-test adjustment.

**Conclusions**—By means of a closed, highly sensitive, and specific real-time PCR, *C. pneumoniae* was not detected in cerebrovascular atherosclerosis. PCR on PBMC was not predictive for endovascular chlamydia infection and most likely stem from previous *C. pneumoniae* respiratory tract infection in individual cases. *(Stroke. 2004;35:000-000.)*

**Key Words:** *Chlamydomphila pneumoniae* ■ polymerase chain reaction ■ senile plaques ■ stroke

Besides conventional risk factors, inflammation seems to play a major role in the pathophysiology of atherosclerosis. During the past years, the probably strongest association between a particular microorganism and atherosclerosis has been postulated for *Chlamydia pneumoniae*, a fastidious, intracellular, Gram-negative bacterium causing repeatedly respiratory tract infections during the lifetime.

Thus, in the field of *C. pneumoniae* and stroke, numerous studies with the attempt to directly identify the organism in the affected tissue were conducted. However, data are contradictory, especially reported positivity rates by means of polymerase chain reaction (PCR), which vary between 0% and 100%. In addition, large interventional trials have not been able to show a beneficial effect of antimicrobial therapy on the clinical outcome in patients with coronary heart disease to date. More than 10 years after the first association between the pathogen and atherosclerosis was reported, there is still no evidence for a causal relationship, and the controversial scientific discussion is ongoing, thereby separating researchers of many medical fields.

Diagnosing *C. pneumoniae* infection is fastidious, nonstandardized, and nonvalidated. This is true for all detection methods available inclusive of PCR. Irrespective of patient collectives studied or clinical questions posed in definite trials, unfortunately, there is evidence of major problems with both interlaboratory and intralaboratory reproducibility of PCR. Much of the variation in the prevalence of *C. pneumoniae* DNA detected in human atheroma specimens reported in the literature is probably caused by variation/shortcomings of methods such as nested PCR technology, which is highly prone to produce false-positive results.

In contrast to already published studies in the field of *C. pneumoniae* and stroke, this, to our knowledge, is the first study applying quantitative real-time PCR technology, which today is the state-of-the-art diagnostic PCR technique. Thus, the aims of this study were to investigate in symptomatic and...
asymptomatic patients with severe cerebrovascular atherosclerosis by means of real-time PCR technology, (1) the prevalence of C. pneumoniae DNA in carotid arteries and peripheral blood (to investigate whether the latter could be used as a marker to predict endovascular chlamydial infection), (2) the "chlamydial load" of tissue and blood specimens, and, dependent on the chlamydia status, and (3) whether there is a correlation between patients groups and C-reactive protein (CRP) levels.

Materials and Methods

Patients

Between March 1999 and May 2001, all patients with hemodynamically significant carotid artery stenosis of at least 80% (range 80% to 99%; determined by means of color duplex imaging) who were consecutively admitted for endarterectomy at the Department of Vascular Surgery of the Vienna University Hospital were enrolled. Seventy-five carotid atheroma specimens and peripheral blood mononuclear cells (PBMC) from 75 patients (52 male, mean age 71.8 years, range 48 to 88 years; 23 female, mean age 68.7 years, range 55 to 81 years; 30 patients asymptomatic versus 45 symptomatic [transient ischemic attack, amaurosis fugax, prolonged reversible ischemic neurological deficit, stroke]) were available. Clinical details and patient characteristics with respect to symptoms are displayed in the Table.

Endarterectomy Samples

Within 2 hours after surgery, specimens arrived at the microbiology laboratory. After removal of macroscopically stony parts and fatty tissue, a representative sample (50 mg) comprising the entire vessel wall was resected with a sterile scalpel and homogenized. One half of the homogenate was immediately cultured onto HEp-2 cells for 8 passages as described recently. The other half of the homogenate was stored at −80°C. DNA was then extracted by means of the QIAamp DNA Mini Kit (QIAGEN; tissue protocol) and eluted in a volume of 100 μL. DNA amount between 0.3 and 0.85 μg/10 μL DNA solution as measured by spectrophotometric analysis (VersaFluor, Biorad). One half of the homogenate was resected with a sterile scalpel and homogenized. The other half of the homogenate was immediately cultured onto HEp-2 cells for 8 passages as described recently. The other half of the homogenate was stored at −80°C. DNA was then extracted by means of the QIAamp DNA Mini Kit (QIAGEN; tissue protocol) and eluted in a volume of 100 μL. DNA amount between 0.3 and 0.85 μg/10 μL DNA solution as measured by spectrophotometric analysis (VersaFluor, Biorad). For every 2 specimens, 1 extraction negative control (ENC; 25 mg porcine aortic tissue) was handled at every third position and treated like the clinical samples, which revealed a DNA amount between 0.3 and 0.85 μg/10 μL DNA solution as measured by spectrophotometric analysis (VersaFluor, Biorad).

PBMC

On admission and before endarterectomy, 6 mL EDTA blood was collected. PBMC were prepared within 8 hours using Ficoll-Paque (Pharmacia, Sweden) and resuspended with 40 μL distilled water. DNA was extracted from 20 μL and eluted in 50 μL using the QIAamp DNA Mini Kit (QIAGEN; blood and body fluid protocol). For every 2 patients, PBMC samples from a volunteer were processed and used as ENCs.

Serology and CRP

Preoperative serum samples were tested for the presence of species-specific IgM, IgG, and IgA antibodies against C. pneumoniae using a microimmunofluorescence test (MIF) (Labsystems, Finland). CRP values (measured by means of a nephelometric assay by Dade Behring; cutoff: 5 mg/L) were determined within the routinely established laboratory profile of each patient on the day before endarterectomy.

Control Strain of C. pneumoniae and Standards for Real-Time PCR

A local vascular C. pneumoniae isolate was cultured and used to determine the analytical sensitivity of PCR and for spiking carotid tissue as well as PBMCs. Standards for real-time PCR contained

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### Clinical Characteristics, C. pneumoniae Status, and C-Reactive Protein Values of Patients With Hemodynamically Significant Carotid Artery Stenosis (Mean 88%) With Respect to Symptoms

<table>
<thead>
<tr>
<th>Symptomatic</th>
<th>TIA</th>
<th>Amaurosis Fugax</th>
<th>PRIND</th>
<th>Stroke</th>
<th>Total</th>
<th>Asymptomatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>N of patients</td>
<td>19</td>
<td>8</td>
<td>11</td>
<td>14</td>
<td>45*</td>
<td>30</td>
</tr>
<tr>
<td>Mean age ± SD</td>
<td>71.3±6.7</td>
<td>69.9±8.7</td>
<td>69.7±9.6</td>
<td>67±9.0</td>
<td>69.3±8.5</td>
<td>73.1±7.9</td>
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<tr>
<td>Sex, male/female</td>
<td>11/8</td>
<td>7/1</td>
<td>9/2</td>
<td>8/6</td>
<td>28/17</td>
<td>23/7</td>
</tr>
<tr>
<td>N of patients with history of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHD and/or PAD</td>
<td>12</td>
<td>4</td>
<td>6</td>
<td>9</td>
<td>28</td>
<td>15</td>
</tr>
<tr>
<td>Asthma or COPD</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>DM</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>6</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Hypertension</td>
<td>10</td>
<td>3</td>
<td>4</td>
<td>8</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>C. pneumoniae isolated from atheroma</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>C. pneumoniae DNA in atheroma</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C. pneumoniae DNA in PBMC</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mean CRP ± SD</td>
<td>0.32±1</td>
<td>0.68±1.55</td>
<td>0.90±1.5</td>
<td>0.69±1.26</td>
<td>0.45±1.0</td>
<td>0.40±1.18</td>
</tr>
<tr>
<td>N of patients with C. pneumoniae-specific antibodies (reciprocal titer):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IgM (16)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2 (borderline)</td>
</tr>
<tr>
<td>IgG (≥32)</td>
<td>14</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>31</td>
<td>24</td>
</tr>
<tr>
<td>IgA (≥8)</td>
<td>14</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>33</td>
<td>18</td>
</tr>
</tbody>
</table>

*Six patients were counted for >1 symptom category (TIA, amaurosis, PRIND, stroke).

TIA indicates transient ischemic attack; PRIND, prolonged reversible ischemic neurological deficit; CHD, coronary heart disease and/or myocardial infarction; PAD, peripheral arterial disease; COPD, chronic obstructive pulmonary disease; PBMC, peripheral blood mononuclear cells; CRP, C-reactive protein.
salmon testis carrier DNA and were in a range between $10^6$ and $10^{-1}$ elementary bodies (EBs)/$10 \mu L$ eluted DNA. Exhaustive care was taken to avoid contamination.}

**Quantitative Real-Time PCR TaqMan Assay**

The *C. pneumoniae* specific sequences of primers and probe for the TaqMan-based real-time PCR assay were selected from the *ompA* gene of *C. pneumoniae*. Data in context with sensitivity, specificity, and reliability, as well as a comparison with the 4 most widely used conventional PCRs for detection of *C. pneumoniae*, were recently published. 

Real-time PCR was performed in 96-well MicroAmp optical plates (Applied Biosystems) and optical adhesive covers with reaction volumes consisting of 2.5 $\mu L$ of the Brilliant Quantitative PCR Core Reagent Kit (Stratagene), each of the primers at a concentration of 300 nM, 200 nM TaqMan probe, and 10 $\mu L$ of template DNA (standards, PBMC, or endarterectomy samples) in a total reaction volume of 25 $\mu L$. Three replicates of each dilution of the standards, the PBMC or carotid extracts, were amplified. Amplification, detection, and quantification of amplicon was performed by an ABI Prism 7700 sequence detection instrument (Applied Biosystems) as described recently. 

In a separate session, a fourth replicate of each clinical sample was checked for PCR inhibitors by comparing the amplification plot of the sample spiked with DNA corresponding to 20 EBs to that using DNA of 20 EBs as a template alone. Inhibited specimens were retested as 1:10 diluted. PCR analyses were considered negative for *C. pneumoniae* DNA if the $C_T$ values exceeded 40 cycles.

**Statistical Analysis**

All statistical analyses were performed by means of SPSS V.7.5.1 (SPSS Inc.). For comparison of patient groups with *C. pneumoniae* PCR results, Fisher exact test, and CRP values, the 1-way ANOVA test were applied and corrected for multiplicity-of-test adjustment. $P$ $\leq$ 0.05 was considered statistically significant.

**Results**

Not one of 75 carotid endarterectomy samples yielded *C. pneumoniae* DNA, and we were unable to isolate the organism. All real-time PCR analyses were performed in triplicate. Plaques and PBMC spiked with DNA corresponding to 20 EBs revealed inhibition in 8 of 75 (11%) and 12 of 73 (16.4%) specimens, respectively. Thus, these samples were retested as 1:10 diluted (spiked and unspiked ones). The resulting increase of the curves given by the amplification plots (in the background of sufficient DNA) then indicated successful detection of 2 EBs (spiked sample), thereby making inhibition (and thus false-negative PCR results) very unlikely and should allow for detection of low target numbers in the unspiked sample. Re-analyzed specimens remained negative for *C. pneumoniae* DNA. All ENCs and no template controls were correctly identified as negative, and amplification plots of the standards reacted as expected.

Four of 75 (5.3%) patients (all male; mean age 71 years), 2 of whom were symptomatic (transient ischemic attack; amaurosis fugax) had detectable *C. pneumoniae*-DNA in their preoperative PBMC samples. As determined by quantitative real-time PCR, the mean $C_T$ values of 3/3, 2/3, 2/3, and 1/3 replicates testing positive were 37.6, 35.5, 36.1, and 37.3, respectively. The numbers of corresponding EBs were 8, 65, 35, and 8 per PCR reaction, which was <1 bacterium, or, more precise, 1 inclusion (consisting of ~400 EBs) present in 6 mL EDTA blood. CRP values measured in all 4 patients were <0.5 mg/dL and all patients had *C. pneumoniae*-specific IgG and/or IgA antibodies. *C. pneumoniae*-specific IgM, IgG, and IgA antibodies were detectable in 1 of 74 (1.4%); titer: 1:16; 3 patients borderline, 55 of 74 (74%), and 51 of 74 (69%) cases. Forty patients had both IgA (titer $\geq 1:8$) and IgG (titer $\geq 1:32$) antibodies. Fifteen (37.5%) and 29 (72.5%) of these were asymptomatic and had normal CRP values (<0.5 mg/dL), respectively. Only 11 patients of those with IgG and IgA antibodies against *C. pneumoniae* showed elevated CRP values (mean, 1.6 mg/dL; SD $\pm$ 1.2; range, 0.6 to 4.4 mg/dL).

Irrespective of anti-*C. pneumoniae* antibody titers in the individuals studied, CRP values measured by means of a highly sensitive assay in this study were low (mean, 0.44 mg/dL; SD $\pm$ 1.1; range, < 0.5 mg/dL to 6.1 mg/dL). Fifty-eight of 75 (77%) patients had CRP levels <0.5 mg/dL. The Table summarizes clinical characteristics, *C. pneumoniae* PCR results on atheroma and PBMC, *C. pneumoniae* serological results, and CRP values with respect to symptoms.

No significant correlation was found between patient groups (symptomatic versus asymptomatic), *C. pneumoniae* PCR results and CRP values by means of Fisher exact test and by 1-way ANOVA after correction for multiplicity-of-test adjustment, respectively.

**Discussion**

During the past years, numerous articles have been published studying a possible association of *C. pneumoniae* and various aspects in context with the development of cerebrovascular atherosclerosis by means of the “nested” PCR technique. Some authors found a correlation between *C. pneumoniae* DNA in atheroma and peripheral blood and, thus, suggested that *C. pneumoniae* PCR on PBMC might be useful as a marker to predict endovascular chlamydial infection. Others, however, suggested a patchy distribution of *C. pneumoniae* within the atherosclerotic plaque.

Unfortunately, *C. pneumoniae* PCR is not standardized. Recently, it has been shown that conventional PCR techniques, particularly nested ones (2 PCR reactions running subsequently), pose problems and are the major reason for false-positive PCR results. There is no consensus on how (if at all) nested PCR technology can be controlled. Thus, data generated and conclusions drawn must be interpreted with uttermost caution and care.

When reviewing the literature in the field of *C. pneumoniae* and cerebral atherosclerosis, not even 2 studies would turn out to be comparable in context with PCR methodology applied. Nearly all investigators used outdated PCR technology highly prone to produce false-positive results and did not confirm their positive findings. Farsak et al and Prager et al, for instance, found *C. pneumoniae* DNA by conventional PCR (described by Gaydos et al in 1992) in 44.4% and 82% of carotid–atheroma specimens, respectively. Since 1992, this protocol has been repeatedly modified and optimized. Despite that, this method did not detect >1 *C. pneumoniae* DNA-positive coronary artery plaque out of a total of 56 specimens in the Gaydos laboratory. By means of nested PCR, Sessa et al reported carotid atheroma, PBMC, and even lymph nodes
of stroke patients to contain \( \textit{C. pneumoniae} \) DNA in 18 of 51 (35\%), 23 of 51 (45\%), and 12 of 51 (23.5\%), respectively.

This, to our knowledge, is the first study applying real-time PCR technology on carotid endarterectomy specimens and PBMC of patients with severe cerebrovascular atherosclerosis. This new PCR technique offers numerous advantages such as a closed, automated, non-nested system including amplicon inactivation. Hence, once a PCR reaction is setup, manipulations with a potentially highly positive liquid become unnecessary. Thus, the chance to produce false-positive results is dramatically abated. Furthermore, these advantages are offered without losing sensitivity or specificity, but with the additional option to quantify the PCR product and to detect inhibition.

We did not detect \( \textit{C. pneumoniae} \) DNA in a single carotid endarterectomy specimen from 75 patients with severe, mostly generalized atherosclerosis. In addition, and although we succeeded in culturing \( \textit{C. pneumoniae} \) from atherosclerotic vessels in a study conducted earlier, we failed in doing so in the present one.\(^{12} \) Patients definitely did not receive perioperative prophylaxis. Unfortunately, we do not have information in context with preceding respiratory tract infections or previously administered “antichlamydia” therapy. Even though growth of \( \textit{C. pneumoniae} \) might have been constricted, at least DNA of the organism should have been detectable by the real-time PCR. As a consequence of a varying annual incidence of \( \textit{C. pneumoniae} \) respiratory tract infection\(^{7} \) detection within plaques might probably vary, too.

False-negative PCR results caused by the presence of PCR inhibitors were not very likely, because each clinical sample was checked for inhibitors. Moreover, the real-time PCR assay applied in the present article was already extensively evaluated in terms of reproducibility, sensitivity, and specificity and was compared with cell culture and other widely used conventional PCR assays for detection of \( \textit{C. pneumoniae}. \)\(^{9} \) In addition, the assay was subjected to an external quality-control study\(^{20} \) and proved to produce highly sensitive, specific, and reproducible, thus highly reliable, results.

There was no significant difference in age, gender, patient history, CRP levels, or antibody titers against \( \textit{C. pneumoniae} \) for symptomatic and asymptomatic patients. In most patients, >1 vascular region was affected by severe atherosclerosis. With focus on a generalized, chronic inflammatory component of atherosclerosis and results reported by others,\(^{14,21} \) we would have expected much higher CRP levels. However, in 4 patients, \( \textit{C. pneumoniae} \) DNA was detected in peripheral blood drawn before endarterectomy, but chlamydial loads were very low. This finding is in concordance with data published by Tondella et al.,\(^{22} \) who found only 1 of 228 (0.4\%) PBMC of stroke patients to be positive for \( \textit{C. pneumoniae} \) DNA by real-time PCR, at a copy number of 1.3 in 2 of 5 replicates tested. In addition, Smieja et al.\(^{23} \) published data on replicate testing, which might be necessary not to underestimate the true prevalence of \( \textit{C. pneumoniae} \) DNA-positive PBMCs. They found that testing 1, 3, or 5 replicates detected 3, 5, or 9 of 10 true \( \textit{C. pneumoniae} \) DNA-positive PBMC samples. With this in mind, and being well-aware of the pitfalls of conventional PCR, it is not astonishing that others\(^{14,15,24,25} \) reported PBMC positivity ratios in stroke patients up to 87\% by means of nested PCR without repetitive testing at all.

Because all 4 \( \textit{C. pneumoniae} \) DNA-positive PMBC patients had detectable species-specific antibodies against \( \textit{C. pneumoniae} \) (2 patients had IgG and IgA), we can safely assume previous exposure to the pathogen. However, this is not surprising in the age group studied here (as well as in studies already published in this field). \( \textit{C. pneumoniae} \) respiratory tract infection is a common event among the elderly population\(^{26} \) and seems to be the most likely clinical correlate and explanation accounting for, in some cases, \( \textit{C. pneumoniae} \) DNA detected in PBMC and, in other cases, the antibody titers measured in our patients. How to distinguish in the individual case whether \( \textit{C. pneumoniae} \) DNA detected in PBMC could have originated from either the respiratory tract or the vascular system is unknown. In the present study, however, such a differentiation was needless because of the fact that all carotid atheroma were \( \textit{C. pneumoniae} \) DNA-negative.

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References


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