Is the Perceived Association between \textit{Chlamydia pneumoniae} and Vascular Diseases Biased by Methodology?

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Inter- and intralaboratory inconsistencies in detection rates of \textit{Chlamydia pneumoniae} in vascular specimens have been demonstrated. In this study, 66 vascular tissue specimens from 66 patients with vascular disease were tested by three PCR assays: a 16S PCR-based reverse line blot (RLB) assay, a single-step PCR, and a nested PCR. Also, we explored the impacts of different DNA polymerase enzymes on the results based on gel electrophoresis and hybridization. The PCR results by gel electrophoresis in the single-step PCR depended on which DNA polymerase was used. All samples were negative with AmpliTaq Gold DNA polymerase, and 54.5% (36 of 66) were positive with the conventional Taq DNA polymerase. All samples were negative after hybridization with a \textit{C. pneumoniae}-specific probe. In the nested PCR, all specimens were negative by gel electrophoresis and after hybridization. The RLB assay failed to detect \textit{C. pneumoniae} in any specimen; however, 20 specimens were \textit{Chlamydia} sp. positive. The sequence analysis of six of these samples demonstrated \textit{Chlamydia}-like organisms. RLB detected \textit{Chlamydia} sp. DNA in water and in the elution buffer after passage of the Qiagen columns (11 of 40). This study identified factors that may influence the detection of \textit{C. pneumoniae} DNA in vascular tissues and consequently bias the perception of a link between \textit{C. pneumoniae} and vascular diseases. The following are strongly recommended: to use DNA polymerases that have to be activated, to decontaminate with dUTP–uracil-DNA glycosylase, to hybridize with specific probes, to include sufficient controls, and to use molecular grade water.

The association between \textit{Chlamydia pneumoniae} and vascular diseases has gained considerable attention in recent years. Culturing of \textit{C. pneumoniae} is difficult, and only in sporadic cases has the microorganism been successfully isolated. Therefore, molecular detection methods have been widely used to investigate the association between \textit{C. pneumoniae} and vascular diseases. Several PCR assays have been described, and those described by Gaydos et al. (17), Campbell et al. (11), and Tong and Sillis (49) have been most widely used in various investigations. PCR assays for the detection of \textit{C. pneumoniae} are still not standardized, and investigators in the field face many problems, including nonspecific amplification, contamination, and poor sensitivity (9, 10, 15).

Major inter- and intralaboratory inconsistencies in detection rates of \textit{C. pneumoniae} in vascular specimens have been demonstrated recently (3). The same study also showed that many false-positive results were obtained. Several issues have been linked to the variation in detection rates of \textit{C. pneumoniae}, such as specimen collection and processing, DNA extraction, the choice of primers, visualization of PCR products, and false-positive and false-negative signals (9).

In this study, we investigated vascular tissue specimens from patients undergoing surgery by a 16S PCR-based reverse line blot (RLB) assay to detect \textit{C. pneumoniae} DNA, as well as \textit{Chlamydia} sp. DNA. In addition, we performed the PCR assays described by Gaydos et al. (17) and Tong and Sillis (49). Also, we evaluated the impacts of two DNA polymerase enzyme types on the detection of \textit{C. pneumoniae} in vascular tissues.

**MATERIALS AND METHODS**

Vascular tissue specimens were obtained from 66 patients with vascular disease who were undergoing vascular surgery. Sixty-one patients were subjected to surgery because of peripheral atherosclerotic disease, and five patients were subjected to surgery because of abdominal aortic aneurysms. The specimens included 40 atherosclerotic specimens from the femoral artery, 9 specimens from the carotid artery, 7 specimens from the iliac artery, 5 specimens from the popliteal artery, and 5 abdominal aortic aneurysm specimens. The specimens were transported to the laboratory in a Tris-EDTA buffer containing 0.5% sodium dodecyl sulfate. In the laboratory, vascular specimens were stored at −70°C until further processing. The local ethical committee approved the study, and the included patients gave their informed consent.

**PCR.** DNA extraction from specimens was performed using the QIAamp DNA minikit (Qiagen, Hilden, Germany) as described previously (7). Detection of \textit{C. pneumoniae} DNA was carried out by the following PCR assays. Assay A1 was a single-step \textit{C. pneumoniae} PCR targeting the 16S rRNA gene essentially as described by Gaydos et al. (17). Assay A2 was the same as assay A1 with the following modifications: uracil-DNA glycosylase (UNG) was used prior to amplification, dUTP was used instead of deoxynucleoside triphosphates, and AmpliTaq Gold DNA polymerase was used instead of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Assay B1 was a nested \textit{C. pneumoniae} PCR targeting the ompA gene, essentially as described by Tong and Sillis (49). Assay B2 was the same as assay B1 but using AmpliTaq Gold DNA polymerase instead of Taq DNA polymerase. Assay C was an in-house \textit{Chlamydia} sp. PCR targeting the 16S rRNA gene. Assay D was a \textit{Chlamydia} sp. PCR targeting the 16S rRNA gene, as described by Ossewaarde and Meijer (41). Assay D was performed only...
for identification by sequencing. The primers and probes used in assays A to D are listed in Table 1.

To minimize the risk of contamination and to prevent carryover from previous PCRs, dUTP and UNG were used in all assays except assay A1 and the nested PCRs (assays B1 and B2). Sample preparation, preparation of PCR mixtures, and PCR assays were performed in separate rooms. In all PCR runs of each assay, positive controls in dilution series were included to monitor sensitivity. This was accomplished by spiking five known concentrations of C. pneumoniae DNA (range, 0.01 to 100 inclusion-forming units [IFU]) in a pool of negative clinical material. Purified C. pneumoniae DNA (strain TW-183) was kindly provided by the Research Laboratory for Infectious Diseases, National Institute of Public Health and the Environment, Bilthoven, The Netherlands. In assay C, we used a clinical sample positive for Chlamydia trachomatis for the Chlamydia trachomatis genus-, the C. pneumoniae species-specific, and the C. trachomatis species-specific probes.

Negative controls were included after every four samples during processing and PCR. PCRs were performed in a PE 9600 Thermocycler (Perkin-Elmer Cetus) with the following settings.

(ii) Assays A1 and A2. The PCR mixture for assays A1 and A2 contained 30 pmol of each primer, 1.5 mM MgCl₂, 200 µM dUTP, 2.5 U of Taq DNA polymerase (assay A1) or AmpliTaq Gold DNA polymerase (assay A2), and 5 µl of extracted DNA. For assay A2 only, 0.25 U of UNG was used. The final reaction volume was 25 µl. The PCR program was as follows: 2 min at 50°C, 10 min at 96°C (only for assay A2), followed by 40 cycles of 30 s at 95°C, 1 min at 55°C, and 1 min at 72°C. A final step of 10 min at 72°C completed the PCR.

(ii) Assays B1 and B2. The first PCR mixture for assays B1 and B2 contained 10 pmol of each external primer, 1.5 mM MgCl₂, 200 µM dUTP, 2.5 U of Taq DNA polymerase (assay B1) or AmpliTaq Gold DNA polymerase (assay B2), and 10 µl of extracted DNA in a final volume of 25 µl. The first PCR program consisted of 10 min at 96°C, followed by 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C. For 22 cycles, the annealing temperature was lowered 1°C every two cycles to 55°C. Subsequently, 20 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C were performed. The second PCR mixture contained 25 pmol of each internal primer, 3 mM MgCl₂, 200 µM dUTP, 2.5 U of Taq DNA polymerase (assay B1) or AmpliTaq Gold DNA polymerase (assay B2), and 10 µl of extracted DNA in a final volume of 25 µl. The first PCR program consisted of 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The second PCR product consisted of 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The third PCR mixture contained 20 pmol of primer CHLF, 10 pmol of primer CHLR-B, 12.5 µl of Hotstar-MasterMix (Qiagen). Five microliters of template DNA was added to the mixture, and the final volume was 25 µl. Samples were subjected to the following PCR program: two cycles of 20 s at 95°C, 1 min at 66°C, and 1 min at 72°C. Subsequently, every two cycles, the annealing temperature was lowered by 2°C to 56°C, followed by 40 cycles of 20 s at 96°C, 1 min at 56°C, and 1 min at 72°C. The PCR was completed with 7 min at 72°C.

(iii) Assay C. The PCR mixture for assay C contained 20 pmol of primer CHLF, 10 pmol of primer CHLR-B, 12.5 µl of Hotstar-MasterMix (Qiagen). Five microliters of template DNA was added to the mixture, and the final volume was 25 µl. Samples were subjected to the following PCR program: two cycles of 20 s at 95°C, 1 min at 66°C, and 1 min at 72°C. Subsequently, every two cycles, the annealing temperature was lowered by 2°C to 56°C, followed by 40 cycles of 20 s at 96°C, 1 min at 56°C, and 1 min at 72°C. The PCR was completed with 7 min at 72°C.

(iv) Assay D. The PCR mixture for assay D contained 10 pmol of each primer and 12.5 µl of Hotstar-MasterMix. The PCR program was identical to that of assay C described above.

(v) Visualization of PCR products and interpretation of results. PCR products were visualized after electrophoresis in 2% ethidium bromide-stained agarose gels (MP agarose; Roche Biochemicals, Indianapolis, Ind.). The PCR products (3 µl) of assay A and assay B were also spotted and hybridized with the appropriate 5’-biotin-labeled probe (Table 1). Hybridization signals were visualized using streptavidin-peroxidase and enhanced chemiluminescence detection reagents (Amersham, Little Chalfont, United Kingdom).

The PCR products of assay C were obtained with a biotinylated primer. These products were hybridized to the Chlamydia sp., C. pneumoniae, and C. trachomatis-specific oligonucleotide probes that were covalently bound to a Biodyne C membrane (Amersham) using a miniblotti (Immunetics, Cambridge, Mass.) in an RLB assay (Table 1 and Fig. 1). The technique of RLB hybridization was previously described (46). In brief, the PCR products were denatured for 10 min at 95°C after dilution of 10 µl of the biotin-labeled PCR product in 130 µl of 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.4])–0.1% sodium dodecyl sulfate. After denaturation, PCR products were added to the membrane and hybridized for 1 h at 42°C. Hybridization signals were visualized using streptavidin-peroxidase and enhanced chemiluminescence detection reagents (Amersham).

PCR runs were judged by negative and positive controls. The results obtained by agarose gel electrophoresis were scored as either PCR positive or PCR negative. PCR samples that gave clear hybridization signals in dot spot or RLB analysis were scored PCR positive and/or Chlamydia sp. positive, according to the probe used.

### Statistical analysis
Statistical analysis was performed with SPSS for Windows version 11.0. A P value of <0.05 was considered statistically significant.

## RESULTS
During surgery, vascular tissue specimens were obtained from 66 patients with vascular disease, 61 patients with peripheral atherosclerotic disease, and 5 patients with abdominal aortic aneurysms. The mean age of the patients was 61 years.

![FIG. 1. Part of the RLB after hybridization with PCR products obtained from controls and clinical specimens. Chlamydia genus- , C. pneumoniae-, and C. trachomatis-specific probes (200 and 100 pmol) were spotted horizontally, as indicated on the left. The PCR products were hybridized vertically. Lane 1, 10 IFU of C. pneumoniae DNA; lane 2, 1.0 IFU of C. pneumoniae DNA; lanes 3 and 7, negative controls; lanes 4 to 6 and 8 to 10, clinical specimens.](http://jcm.asm.org/)
TABLE 2. PCR results in 66 vascular specimens by different PCR assays

<table>
<thead>
<tr>
<th>No. of specimens</th>
<th>Single-step PCR</th>
<th>Nested PCR</th>
<th>PCR-based RLB</th>
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<tbody>
<tr>
<td></td>
<td>A1</td>
<td>A2</td>
<td>B1</td>
</tr>
<tr>
<td>18</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>28</td>
<td>PCR+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>PCR+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>–</td>
<td>–</td>
<td>Cspp+</td>
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* In assays A1 and B1, Taq polymerase was used; in assays A2 and B2, AmpliTaq Gold polymerase was used; in assay C, Hotstar Taq DNA polymerase was used. –, PCR was negative both in agarose gel electrophoresis and after hybridization; PCR+, PCR was positive in agarose gel electrophoresis and negative after hybridization; Cspp+, Chlamydia sp. positive in RLB hybridization. A total of 36 specimens were positive by single-step PCR assay A1, and a total of 20 specimens were positive by PCR-based RLB assay C.

DISCUSSION

The possible role of *C. pneumoniae* in the pathogenesis of vascular disease has been widely investigated. The DNA amplification of *C. pneumoniae*-specific sequences in vascular tissue specimens, however, has been shown to vary greatly among study groups. More recent reports have criticized the lack of standardization of *C. pneumoniae* PCR methodology (3, 4, 15).

In this study, we investigated vascular tissue specimens from 66 patients by the PCR assays that have been most widely used (17, 49), and we explored the impacts of different DNA polymerase enzymes on the results based on agarose gel electrophoresis and after hybridization. In addition, we investigated the presence of *Chlamydia* sp. DNA in the 66 samples, using a *Chlamydia* sp. PCR and RLB hybridization with both a *Chlamydia* sp-specific and a *C. pneumoniae*-specific probe.

The rate of PCR-positive signals by gel electrophoresis in the single-step PCR depended on which DNA polymerase was used. When the conventional *Taq* DNA polymerase was used (assay A1), 36 specimens (54.5%) were PCR positive. If agarose gel electrophoresis had been the final method of detection in combination with the use of *Taq* DNA polymerase, 36 of 66 samples would have been labeled *C. pneumoniae* positive in our study. These 36 samples, however, could not be confirmed by hybridization of the PCR products with a *C. pneumoniae*-specific probe. Also, these samples were negative when AmpliTaq Gold DNA polymerase was used. This may be due to the production of more specific products by AmpliTaq Gold DNA polymerase than by the conventional *Taq* DNA polymerase. DNA polymerase enzymes, like AmpliTaq Gold or Hotstart, that need to be activated at elevated temperature are known to enhance the specificity of a PCR assay (27). Reviewing the literature on the use of AmpliTaq Gold DNA polymerase in *C. pneumoniae* PCR assays, we found that in the majority of the studies, the type of DNA polymerase enzyme used was not identified. In a study that explicitly mentioned the use of AmpliTaq Gold DNA polymerase, the detection rate of *C. pneumoniae* DNA was 14.8% (30).

Hybridization is also an important measure to minimize false PCR-positive signals, and the advantages of confirmation of PCR-positive signals by hybridization with a specific probe have been described before (4). Analyzing 33 studies with regard to hybridization in the detection of *C. pneumoniae* DNA, we found 11 studies in which only gel electrophoresis had been used to visualize PCR products (8, 16, 18, 30, 37, 38, 40, 42–45). In the other 22 studies, PCR results were confirmed by hybridization (2, 5, 6, 12, 14, 19, 23, 31, 26, 28, 29, 31–35, 39, 47, 48, 50). D. Taylor-Robinson, G. Ong, B. J. Thomas, M. L. Rose, and M. H. Yacoub, Letter, Lancet 351:1255, 1998). The detection rate was significantly higher in studies with only gel electrophoresis than in studies with hybridization (31.6% [204 of 645] and 24.5% [367 of 1,492]), respectively (P = 0.0009). It is possible that the results of studies without hybridization are confounded by nonspecific PCR-positive signals that are incorrectly interpreted as *C. pneumoniae* positive.

Using *Chlamydia* sp. PCR and RLB hybridization, we detected *Chlamydia* sp. DNA in 30% (20 of 66) of the specimens. Identification by sequence analysis of 6 of 20 PCR-positive samples demonstrated the presence of *Chlamydia*-like organ-
isms, including Endosymbiont acanthamoebae and Neochlamydia hartmannellae.

Chlamydia-like organisms may infect free-living amoebae that are common inhabitants of the aquatic environment. Amoebae may act as reservoirs for these organisms, implying that Chlamydia-like organisms have potential for widespread dissemination (20). They have been detected in nasal mucosa of healthy persons, in bronchoalveolar lavage fluids of patients with respiratory tract infections, and in abdominal aneurysms (1, 13, 22, 41). It has been demonstrated that elementary and reticulate bodies of Chlamydia-like organisms behave, in the life cycle, similarly to the chlamydial elementary and reticulate bodies (21). In addition, analysis has shown the 16S rRNA sequences of eight Chlamydia-like strains to be highly homologous with those of C. pneumoniae, varying from 85 to 87.6% homology (20). The potential pathogenic role of Chlamydia-like organisms has not been established, and there is no evidence that Chlamydia-like organisms are associated with vascular diseases. Furthermore, it is possible that these strains were not originally present in the patients’ specimens, and the positive PCR signals found might be a result of contamination with environmental Chlamydia-like organisms (36, 41).

We demonstrated the presence of Chlamydia sp. DNA by RLB in 11 samples after passage of the Qiagen columns, including elution buffer and distilled water. In light of the homology between Chlamydia-like organisms and C. pneumoniae, one might hypothesize that sequence homology with Chlamydia-like organisms is responsible for positive results in C. pneumoniae PCR assays. This may, in part, explain the inter- and intralaboratory discrepancies in the detection of C. pneumoniae in vascular tissue specimens.

In the nested PCR, all specimens were negative both by gel electrophoresis and by hybridization. Since the nested PCR was based on the sequence of the OmpA gene, a gene that usually shows more sequence variation than the 16S rRNA gene, no effect of the DNA polymerase was found in this assay. The disadvantage of the nested PCR is that no anticontamination with dUTP-UNG can be used. Therefore, it is most sensitive to contamination.

This study identified important factors that may have contributed to bias and once more shows the important influence of methodological factors on the detection of C. pneumoniae DNA in vascular tissues.

In conclusion, we strongly recommend the use of DNA polymerases that have to be activated, the use of dUTP-UNG anticontamination, hybridization with specific probes, the inclusion of sufficient controls, the use of molecular-grade water, and cautious interpretation of results when column-based DNA extraction methods (e.g., the QIAamp Qiagen minikit) are used. Furthermore, this study stresses the biases that nonstandardized methodology may introduce in the context of the possible link between C. pneumoniae and vascular diseases.

REFERENCES


