Is the Perceived Association between Chlamydia pneumoniae and Vascular Diseases Biased by Methodology?

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Inter- and intralaboratory inconsistencies in detection rates of 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 C. pneumoniae-specific probe. In the nested PCR, all specimens were negative by gel electrophoresis and after hybridization. The RLB assay failed to detect C. pneumoniae in any specimen; however, 20 specimens were Chlamydia sp. positive. The sequence analysis of six of these samples demonstrated Chlamydialike organisms. RLB detected 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 C. pneumoniae DNA in vascular tissues and consequently bias the perception of a link between C. pneumoniae and vascular diseases. The following are strongly recommended: to use DNA polymerases that have to be activated, to decontaminate and vascu-

 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 abdominal aortic aneurysms, and five patients were subjected to surgery because of peripheral atherosclerotic disease. 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 C. pneumoniae DNA was carried out by the following PCR assays. Assay A1 was a single-step 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 C. pneumoniae PCR targeting the omp3 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 Chlamydia sp. PCR targeting the 16S rRNA gene. Assay D was a Chlamydia sp. PCR targeting the 16S rRNA gene, as described by Ossewaarde and Meijer (41). Assay D was performed only

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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 processing, 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 supplied 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 \( C. pneumoniae \) positive and/or \( C. trachomatis \) samples, 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 96°C after dilution of 10 \( \mu l \) of the biotin-labeled PCR product in 150 \( \mu l \) of 2x SSPE (18). 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, 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 mini blotter (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 96°C after dilution of 10 \( \mu l \) of the biotin-labeled PCR product in 150 \( \mu l \) of 2x SSPE (18). 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 \( C. pneumoniae \) 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.
(range, 40 to 89 years). Forty-nine patients (74.2%) were males, and 17 (25.8%) were females.

Samples were subjected to PCR assays A1, A2, B1, B2, and C, and the sensitivity of each assay was experimentally monitored in each run by a dilution series of positive controls spiked in negative clinical material. The lowest detection limit was 0.1 IFU for the single-step PCR (assays A1 and A2) and the PCR-based RLB (assay C). The nested PCR of assays B1 and B2 was less sensitive, with 1.0 IFU as the detection limit.

The results of the PCR assays are shown in Table 2. In the single-step PCR, 36 specimens were PCR positive when *Taq* polymerase was used (assay A1), and none of the specimens was PCR positive when AmpliTaq Gold polymerase was used (assay A2). However, none of the specimens that were PCR positive in the gel electrophoresis could be confirmed after hybridization with a *C. pneumoniae*-specific probe.

In the nested PCR (assays B1 and B2), all specimens were PCR negative, and they were also negative after hybridization. The RLB (assay C) failed to detect *C. pneumoniae* in any specimen; however, 20 specimens were *Chlamydia* sp. positive (Fig. 1). To further specify the *Chlamydia* spp. found, six of the samples were subjected to PCR assay D, and the 16S rRNA gene fragments obtained were sequenced. Using the BLAST server, the sequences obtained were compared with the sequences in GenBank. The sequence analysis demonstrated that these strains (*Chlamydia*-like strains with sequences not present in GenBank) were *Chlamydia* Research Group 52 (one strain), *Chlamydia* Research Group 1 (one strain), *Neochlamydia hartmannellae* (two strains), and *Parachlamydia acanthamoebae* (two strains).

To explore the possibility that these *Chlamydia*-like strains were contaminants, we tested the water (Sigma) used in PCR mixtures, the water after passage of the Qiagen columns, and the elution buffer after passage of the columns. The addition of Sigma water to the PCR mixture of assay C yielded no *Chlamydia* sp.-positive signal. Of the Qiagen column-processed water samples, 6 out of 20, as well as 5 of 20 elution buffer samples, were *Chlamydia* sp. positive.

**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, 23–26, 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. Amebae 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 Chlamydia, 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 Chlamydia, one might hypothesize that sequence homology with Chlamydia-like organisms is responsible for positive results in Chlamydia PCR assays. This may, in part, explain the inter- and intralaboratory discrepancies in the detection of Chlamydia 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 it is not anticontamination with dUTP-UNG can be used. Therefore, it is most necessary to use DNA extraction methods (e.g. the QIAmp Qiagen minikit) are standardized methodology may introduce in the context of the possible link between Chlamydia pneumoniae and vascular diseases.

REFERENCES


