Detection of *C. pneumoniae* by Polymerase Chain Reaction-Enzyme Immunoassay in Abdominal Aortic Aneurysm Walls and its Association with Rupture

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**Objective.** Serological studies have suggested that one of the risk factors for aneurysm development is *C. pneumoniae* infection. The purpose of this study was to evaluate whether there is an association between the presence of *C. pneumoniae* DNA in aneurysms and ruptured Abdominal Aortic Aneurysms.

**Methods.** Aortic walls were collected consecutively from 30 patients with intact AAA, 16 patients with ruptured AAA and 19 healthy organ donors (control). Purified DNAs from all aortas were analyzed for the presence of *C. pneumoniae* DNA in parallel by polymerase chain reaction-enzyme immunoassay (PCR-EIA) and agarose gel electrophoresis. PCR-EIA has a high sensitivity in detecting low DNA copy number in clinical atherosclerotic samples.

**Results.** *C. pneumoniae* DNA was detected more frequently in patients with aneurysms, particular with ruptured aneurysms. The incidence of positive *C. pneumoniae* DNA was 73.3% in intact AAA and 10.5% in control aortas, with the highest frequency in ruptured AAA (100%) (*p*<0.05).

**Conclusion.** Giving the high specificity and sensitivity of PCR-EIA, these findings support the association of *C. pneumoniae* in the pathogenesis of aneurysm development, growth and rupture.

**Keywords:** *C. pneumoniae*; Abdominal aortic aneurysm; Rupture; DNA; PCR-EIA.

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**Introduction**

Inflammation has been found to be an important event in the development and progression of atherosclerosis.1 There is growing evidence that infections with certain pathogens by initiating an inflammatory response might contribute to this disease process. Among some well-known infectious agents such as *Helicobacter pylori* and *cytomegalovirus*, *Chlamydia pneumoniae*, an obligate intracellular bacterium, has been implicated in the initiation of inflammatory response that might contribute to atherosclerosis.2 *C. pneumoniae* has also been suggested as an inflammatory agent responsible for the destruction and weakening of the aortic wall by the release of inflammatory mediators in response to the atherosclerotic plaques.3 Cell biological studies have revealed that Chlamydial heat shock protein is able to stimulate cytokine and metalloproteinase secretion from macrophages and promote a pro-thrombotic endothelial and smooth muscle cell phenotype.4 Therefore, it has been suggested that *C. pneumoniae* might initiate atherosclerosis and subsequently contribute to aneurysm development.

Seroepidemiologic studies have indicated an association between chronic *C. pneumoniae* infection and aneurysmal diseases. The organism has been demonstrated in the serum of more than half of a cohort of abdominal aortic aneurysms (AAAs).5,6 However, Maass et al. strongly suggested that the contribution of *C. pneumoniae* in initiating atherosclerosis requires its presence in the aortas and serology is not useful to identify patients with aortic *C. pneumoniae* infection.7 They found that serology had significantly less sensitivity and positive predictive value for the detection of chlamydial presence as compared with PCR method.7 Several investigators have reported pathogen-specific nonviable structures in atheromatous tissues, although detection rates varied widely with the techniques employed.8-10 Thus, detection of aortic *C. pneumoniae* using a reliable method is required to provide better infection information.
An Italian research group was the first to report an association with AAA and *C. pneumoniae* by detecting *C. pneumoniae* DNA in the atherosclerotic plaques of aortic aneurysms in 1996. In Finland, *C. pneumoniae* DNA was demonstrated in 6 of 6 aneurysm specimens studied by PCR and 6 controls were all negative. The detection of *C. pneumoniae* DNA in ruptured aneurysms was only reported in Poland recently. However, molecular evidence supporting an association between *C. pneumoniae* and AAA rupture in our population is still required.

In 2002, a PCR-based enzyme immunoassay (PCR-EIA) was introduced as a new and reliable tool in molecular diagnosis. It is a hybridization capture assay with high sensitivity, specificity, and a low rate of contamination for DNA detection. In addition, the results can be visualized faster and more sensitively than agarose gel-based PCR assay. This technology has recently been used for detecting *C. pneumoniae* in intestinal mucosal biopsies, with immunohistochemical and nested PCR methods failing to identify *C. pneumoniae* antigen or DNA in the same tissues. Thus, in the present study we aimed to collect both intact and ruptured aortas from patients undergoing aneurysmal repair and evaluate the prevalence of *C. pneumoniae* DNA using this PCR-EIA technique with the novel probe hybridized to a specific sequence of *C. pneumoniae* gene.

**Materials and Methods**

**Patients**

Between January 2002 and December 2003, 30 intact AAA and 16 ruptured AAA specimens without adherent thrombus were collected from consecutive patients who underwent abdominal aortic aneurysmal repair. For control purposes, 19 infrarenal aortic walls were collected from age and sex matched organ donors during transplantation organ procurement for the present PCR-EIA study. All control specimens were macroscopically free from atherosclerotic or vascular diseases. Each specimen was divided into two pieces. One half was snap-frozen in liquid nitrogen within 10 min of collection, and stored in liquid nitrogen until DNA extraction for PCR assay. The remainder was fixed in 10% formalin for 12 h, processed and embedded in paraffin wax for histology. Written informed consent was obtained from all participants, and the protocol was approved by the Ethics Committee.

Histological examination of normal and aneurysmal aortas

Paraffin embedded aortic specimen were cut into 3 μm serial sections and then cleared, rehydrated by sequential immersions in three changes of xylene, followed by graded ethanol and distilled water and then stained with hematoxylin (Merck, Darmstadt, Germany). All sections were then dehydrated and cleared by sequential immersion in gradient ethanol and xylene.

**Tissue processing and DNA extraction**

The DNA extraction from aortic walls was performed according to the standard method with optimized procedures. DNA concentration and purity were estimated spectrophotometrically at optical density (OD) 260/280 using an Eppendorf Biophotometer (Hamburg, Germany).

**Polymerase chain reaction amplification of *C. pneumoniae* DNA**

The presence of *C. pneumoniae* DNA in the aortic walls was determined by Light Diagnostics *C. pneumoniae* OligoDetect® Assay kit (Chemicon, Temecula, CA) with a designed probe hybridized with the nucleotide sequence of *C. pneumoniae* KDTA gene. It is a specific and sensitive polymerase chain reaction-enzyme immunoassay-based method for the qualitative detection of specific *C. pneumoniae* DNA. PCR were performed in a final volume of 50 μl, which included 200 ng DNA extract, 1.25 units of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), 0.2 mM dNTP, 1 mM MgCl₂, and 0.3 μM primers mix. The primers used in the present study were commercially synthesized and provided in the assay kit (Chemicon, Temecula, CA). A 40-cycle amplification was performed consisting of denaturation at 94 °C for 60 s, annealing at 58 °C for 30 s, and extension at 72 °C for 60 s. The denaturing temperature was maintained for 5 min at 94 °C before the first cycle, and the extension temperature was maintained for an additional 8 min after the 40 cycles. Stringent precautions against contamination were taken during the specimen preparation and PCR amplification. The efficiency of DNA extraction and the potential presence of PCR inhibitor(s) in the specimens were monitored by the successful amplification of amplification positive control from the assay kit.
Detection of polymerase chain reaction products by enzyme immunoassay

Amplified products were detected by the assay kit with a 5’ end biotin-labeled internal oligonucleotide probe specific for C. pneumoniae (Chemicon, Temecula, CA). The enzyme immunoassay detection was carried out according to the manufacturer’s protocol. Briefly, 10 μl of PCR products and controls was added to the designated well of the detection plate provided in the assay kit. And then denatured by mixing with 5 μl denaturation solution for 10 min at room temperature. At the end of incubation, 85 μl of hybridization buffer was added. Capture was allowed to proceed for 30 min at 37°C in a water bath, and afterward the wells were washed four times with 250 μl of washing buffer at room temperature. This was followed by the addition of 100 μl of ready to use Strept: HRP Conjugated. The plates were incubated for 30 min at 37°C with mixing, and the wells were washed four times. A total of 100 μl (TMB/E) substrate solution was added, and the plate was incubated for 10 min at 37°C. The OD of the color reaction was measured at 450 nm with an automated ELISA reader. The run was considered valid if all negative control values were less than 0.15 OD units and the positive control values were greater than 1.0 OD units. The background cut-off value was calculated as the average of all negative-control OD values plus 2 standard deviations (SD). The positive cut-off value was calculated by adding the average with 3 SD. A mean OD value from duplicate tests was obtained from each sample. A specimen was considered positive if the mean OD value was greater than the background cut-off value.

Detection of the amplification products electrophoretically on ethidium bromide-stained agarose gels

An aliquot of 8 μl was withdrawn from each PCR reaction tube, resolved in 2% agarose gels in 1× TBE buffer, and visualized by ethidium bromide. The C. pneumoniae amplification positive control and C. pneumoniae DNA positive samples yielded a biotinylated product 100 base pairs (bp) and 110 bp products in length, respectively.

Statistical analysis

The C. pneumoniae DNA detection rate of each group was compared by chi-square test. A p value of <0.05 was considered statistically significant.

Results

Demographic data of AAA patients and healthy organ donors are shown in Table 1. Of the 30 intact AAA patients, 22 (73.3%) were males and with a mean age 69.5 ± 10 years (range 49–77 years). Twelve (75%) of the 16 ruptured AAA patients were males and with a mean age 72.1 ± 7 years (range 50–79 years). The control groups consisted of 14 men (73.7%) and with a mean age 65.5 ± 8 years (range 50–71 years). The mean aneurysm diameter of the intact AAAs and ruptured AAAs were 6.74 ± 1 and 7.03 ± 0.71 cm, respectively. Age, gender, and size of aneurysms did not differ among AAA patients groups. All aneurysmal aortas revealed typical fibrosis of the intima with thickening, atherosclerotic plaques with cholesterol clefts, foam cells and infiltration of lymphocytes (Fig. 1).

The highest C. pneumoniae DNA positive rate was found in ruptured AAA (100%) as compared with 73.3% of intact AAA and 10.5% in control aortas, p < 0.05. There was no significant relationship between age, gender and size of aneurysm and C. pneumoniae DNA detection.

The presence and mobility of PCR products was also determined by the agarose gel electrophoresis. Positive PCR-EIA results from any diseased aortas (except one intact and two ruptured aneurysms) and control aortas were reproduced in the gel electrophoresis with faint bands. The PCR amplification of C. pneumoniae gene produced the expected 110 bp band for aortas from ruptured AAA patients, indicating the presence of C. pneumoniae DNA in ruptured aneurysmal walls (Fig. 2). Representative bands for positive

Fig. 1. Aneurysmal artery revealed typical atherosclerosis with cholesterol clefts, infiltration of lymphocytes and foamy macrophages (stained with hematoxylin, ×400 magnification).
C. pneumoniae PCR results in intact AAA and negative PCR result for control aorta were also shown.

**Discussion**

Atherosclerosis and its related disease, abdominal aortic aneurysm (AAA), present a common vascular condition with life-threatening implications. Rupture of aneurysm is frequently lethal, and accounts for a large percent of the morbidity and mortality. Differences in the conventional cardiovascular factors, such as smoking, diabetes mellitus and hypercholesterolemia do not entirely account for the prevalence or severity of AAA. Therefore, there is intense research interest focused on seeking other atherogenic risk factors. Increasing serological evidence supports an association between C. pneumoniae and the pathogenesis of AAA. In the present study, we evaluated the presence of C. pneumoniae DNA directly in aortic walls obtained from intact AAA patients, ruptured AAA patients and organ donors using a sensitive PCR-EIA technique to amplify a portion of gene specific for C. pneumoniae. Of the 19 control aortas, only two were DNA positive suggesting a non-frequent presence of the pathogen in the aortas of the organ donors. We found that most AAA specimens housed C. pneumoniae DNA. The relatively high presence of this bacterium is probably the result of persistent infection. Metabolically inactive or killed organisms are rapidly eliminated from humoral immune system, as opposed to actively growing C. pneumoniae. In addition, extrinsic DNA is usually rapidly degraded by restriction endonucleases, so the present finding can be considered as an evidence for the presence of viable pathogens in the diseased aortas.

C. pneumoniae DNA was specifically associated with ruptured aneurysms in the present study. A previous report showed that the annual expansion rate of small aneurysms correlated positively with the serum levels of IgA-antibodies against C. pneumoniae. Tambiah et al. showed in an animal model that C. pneumoniae antigen stimulated the dilatation of the abdominal aorta. In addition, the presence of C. pneumoniae had been found to contribute to plaque instability and lead to fissuring. As rapidly expanding aneurysms are more susceptible to rupture, the present finding imply that C. pneumoniae infection may be a factor influencing the expansion rate as well as eventual aneurysm rupture.

Although the pathogenesis of abdominal aortic aneurysm remains largely obscure, inflammation mediated tissue proteolysis has been found to be important in aneurysmal dilatation. The presence of C. pneumoniae might worsen tissue damage by initiating inflammation and exacerbating atherogenesis. Firstly, experimental aortic inflammation and dilatation can be provoked by C. pneumoniae infection. Secondarily, infected vascular cells can produce matrix-degrading metalloproteases, which are important in the degradation of connective tissue in AAA. In addition, C. pneumoniae is able to multiply in
macrophages, vascular endothelial cells in vitro and aortic smooth muscle cells. Chronic C. pneumoniae infection has also been shown to induce a more atherogenic lipid profile by elevating plasma triglyceride and total cholesterol and reducing high-density lipoprotein (HDL)-cholesterol. Hence it could be assumed that C. pneumoniae may play a role in initiating inflammatory response and triggering the development of aortic aneurysms. How C. pneumoniae gain access to the arteries is still unclear, but infected monocytes/macrophages are thought to be potential vectors for dissemination.

The routine laboratory method for the detection of C. pneumoniae infection is serological assay. However, the value of serology has been questioned due to the lack of specific antibodies in sera of patients from whom the organism could be isolated. In addition, serological diagnosis is hampered by the slow antibody response to C. pneumoniae. Ericson found that the degree of immune response was not a predictor of the extent of infection, however, intra-arterial detection of C. pneumoniae was related to the severity of the cardiovascular diseases.

In addition to seroepidemiologic studies, a possible etiologic link has also been investigated through the demonstration of an organism in atherosclerotic lesions using isolation in tissue culture, immunohistochemistry (IHC) and polymerase chain reaction (PCR). However, C. pneumoniae is difficult to recover from clinical samples. Generally, immunological detection finds more evidence for C. pneumoniae than PCR. These uncertainties make it difficult to estimate the value of serology has been questioned due to the slow anti-immunoglobulin response and long-term prognosis of atherosclerosis. Since there is no cross-reactivity in blood vessels, C. pneumoniae DNA in agarose gel negative aortas, despite the fact that both expression techniques were amplifying the same DNA fragment.

Since C. pneumoniae are susceptible to antimicrobial therapy, its potential role in the development of atherosclerosis and aortic aneurysms might result in a radical change in current clinical practice. The initiation of the atherosclerotic lesion as well as the promotion of existing vascular damage by C. pneumoniae infection is certainly conceivable. Further experimental work is required for investigating its etiologic role in vascular pathology.

Presently, PCR combined with EIA hold promise as useful molecular tools for the detection of C. pneumoniae DNA in aneurysmal samples. Our results add to the controversy regarding the role of C. pneumoniae in AAA development.21,32 Our findings suggest that chronic chlamydial infection could accelerate the rate of aneurysmal growth and lead to rupture. Quantitative and time course analysis of C. pneumoniae may be needed to support this claim. As antibiotics treatment against C. pneumoniae is available, further studies are needed to determine the significance of aortic infection in aneurysm development and expansion.

References

Detection of CP by PCR-EIA in AAA

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