Failure to Demonstrate \textit{Chlamydia pneumoniae} in Symptomatic Abdominal Aortic Aneurysms by a Nested Polymerase Chain Reaction (PCR)

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Objective: To investigate whether \textit{Chlamydia pneumoniae} is present in symptomatic abdominal aortic aneurysms (AAA).

Method and materials: After optimisation of DNA extraction procedures an inhibitor-controlled nested polymerase chain reaction (PCR) amplifying fragments of the gene encoding the \textit{C. pneumoniae} specific major outer membrane protein was performed on 124 wall-specimens from 20 patients with symptomatic AAA.

Results: None of the specimens contained \textit{C. pneumoniae}-specific DNA. Minor inhibition of the PCR was noticed especially in media specimens.

Conclusion: Using a sensitive and specific nested PCR, we were not able to detect \textit{C. pneumoniae} in symptomatic AAA. The failure to detect \textit{C. pneumoniae} in symptomatic AAA, combined with previously reported positive findings in atherosclerotic lesions, supports the hypothesis that AAA and atherosclerosis might be two different disease entities.

Key Words: Abdominal aortic aneurysm; Natural history; Pathogenesis; Polymerase chain reaction (PCR); \textit{Chlamydia pneumoniae}.

Introduction

The fundamental cause of abdominal aortic aneurysms (AAA) is unknown. Seventy-five percent of all AAA are diagnosed in males, and are associated with atherosclerotic manifestations, chronic obstructive airway disease, smoking. Despite a familiar tendency of AAA genetic causes of AAA are only anecdotal. Studies of the walls of AAA have shown infiltrates, dominated by neutrophil leukocytes and macrophages. It therefore seems reasonable to assume that the degeneration of the aortic wall may be caused by autolysis due to proteases from the neutrophil granulocytes. Activation and presence of such granulocytes might be caused by a chronic infection.

A correlation between serological evidence of \textit{Chlamydia pneumoniae} infection and atherosclerotic vascular disease has previously been demonstrated, and some authors have demonstrated the same bacterium by polymerase chain reaction (PCR), immunocytochemistry, and electron microscopy in coronary atheroma, while others could not confirm this. Recently, Ong et al. found \textit{C. pneumoniae} DNA by PCR in atherosclerotic lesions from the aorta, iliac, and femoral arteries. \textit{Chlamydia pneumoniae} may infect vascular tissue where the lipopolysaccharide of \textit{Chlamydia sp.} may have powerful effects on the vascular tissue by inducing cytokines.

Thus, \textit{C. pneumoniae} might be implicated in the development of AAA, and we therefore investigated if \textit{C. pneumoniae} could be demonstrated in the aortic wall of symptomatic AAA by use of an inhibitor-controlled, nested PCR.

Material and Methods

Validation of assay

The sensitivity of the PCR–TRF was evaluated by serial dilutions of a known amount of purified \textit{C. pneumoniae} DNA and by performing the cell culture and PCR–RTF in parallel on serially diluted \textit{C. pneumoniae} organisms as previously described for \textit{C. trachomatis}. The specificity of the \textit{C. pneumoniae} PCR–RTF was evaluated by...
testing C. trachomatis DNA, C. psittaci DNA, and clinical isolates of, among others, Mycoplasma pneumoniae, Legionella pneumophila, Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae, Klebsiella pneumoniae, Branhamella catarrhalis, Pseudomonas aeruginosa, Mycobacteria sp., and Candida albicans.

The loss of DNA during extraction and the inhibition of PCR due to unremoved inhibitors after extraction were assessed by use of a previously described C. trachomatis PCR–TRF which provides log-linear quantitative measurements of DNA in the range of 10^6 to one C. trachomatis IFU. Equally, amounts of C. trachomatis L2 DNA corresponding to approximately 10^1 IFU was added to Solution A (TE-buffer), Solution B (TE-buffer that subsequently were subjected to the extraction procedure), and to each of the patient samples after completion of the extraction procedure. The arbitrary loss of DNA was assessed by subtracting the PCR–TRF-signal of Solution B from the PCR–TRF-signal of Solution A. Loss of DNA during the extraction procedure was evaluated for the following preparation procedures: phenol/chloroform extraction, proteinase K treatment, Chelex Resin (Bio-Rad Laboratories), guanidinium thiocyanate (Sigma Chemical Co), Amplicor HCV specimen preparation kit (Roche Diagnostic Systems), G-NOME DNA (BIO 101, Vista), QIAamp Kit (Qiagen Inc). The influence of remaining inhibitors after the extraction procedure was assessed by subtracting the PCR–TRF-signal of the patient sample from that of Solution B. If the reduction in PCR–TRF-signal exceeded 50%, inhibition was said to occur. Negative controls consisted of TE-buffer. Positive controls consisted of C. pneumoniae VR1310.

Sample preparation

During the period 31 October 1994 to 31 October 1996, material from 20 patients acutely operated for asymptomatic AAA were sampled. During the operation a sample from the AAA-wall was taken, and immediately frozen at −20 °C for up to 3 months and hereafter at −80 °C for up to 1.5 years. The samples were thawed and intima were macroscopically separated from media and adventitia with a disposable scalpel and 0.5 cm² specimens of intima and media were made. In order to minimise inhibition of the PCR, additional 0.5 cm² samples from media and intima were divided in five approx. 0.2 cm² small specimens in 10 of the patients. The specimens were subsequently frozen with liquid nitrogen, and homogenised mechanically in separate mortars using sterile disposable utensils. Hereafter DNA were extracted by use of phenol/chloroform according to Maniatis. The specimens were extracted once with phenol, once with phenol/chloroform and twice with chloroform. The extracted DNA was then precipitated with the double amount of ethanol and 1/10 vol 3M NaAc. The precipitate was then washed with 70% ethanol. The samples were dried in a vacuum centrifuge (VR-1, Heto Lab. Equipment, Alleroed, Denmark), and the DNA was resuspended in 200 µl TE-buffer, of which 38 µl was used for analysis of C. pneumoniae and 38 µl was used for analysis of inhibition.

Assays

The primers were derived from the gene encoding the Major Outer Membrane Protein 1, and all analyses were performed in a Perkin Elmer 9600 thermocycler. The first primers used were: 5'-TACTGGATCCCGCTTGCTGAAAAACTATACTAC-3' (Cp OUT A; bp 267 to bp 296) and 5'-CTGGTCTGCTACGCCAGGTCTTGTT-3' (Cp OUT B; bp 763 to bp 740). The first PCR was carried out in a total volume of 50 µl consisting of 38 µl prepared sample, 10 µl buffer [250 mM KCl, 50 mM Tris pH 8.4, 12.5 mM MgCl₂, 0.1 mg of gelatin per ml] and 2 µl of a solution giving total concentrations of: 200 µM of each of dATP, dTTP, dGTP, dCTP (Boehringer Mannheim), 1 µM of each primer C p OUT A and C p OUT B and 1 U/50 µl AmpliTaq DNA Polymerase (Roche Diagnostic Systems). The first PCR was run as follows: a temperature of 94 °C was kept for 15 s, hereafter 20 cycles each consisting of 94 °C for 10 s, 50 °C for 30 s and 72 °C for 45 s were run. Finally the specimens were kept at 72 °C for 10 min.

Second PCR. The primers used were 5'-GTAAGAGACCTAAACCGCCTACAAAT-3' (Cp IN A; bp 301 to bp 330) and 5'-TAGTACCTTTAACCTCCGAATAACCAACGA-3' (Cp IN B; 490 to bp 461). The one primer was labelled with eiruoptum at the 5' end and the other with biotin. Second PCR was carried out in a total volume of 50 µl using 1 µl of specimens processed by the first PCR as template. The final concentration of the 50 µl for second PCR was: 200 µM of each of dATP, dTTP, dGTP, dCTP (Boehringer Mannheim), 1 µM of each unlabelled primer or 0.06 µM of each labelled primer, 50 mM KCl, 10.2 mM Tris, 2.5 mM MgCl₂, 0.02 mM EDTA, 0.1 µg of gelatin per ml and 1 unit AmpliTaq DNA Polymerase (Roche Diagnostic Systems, Basel, Switzerland). Second PCR was performed as follows: a temperature of 94 °C was kept for 15 s; hereafter 35 cycles each consisting of 94 °C for 10 s, 50 °C for 30 s and 72 °C for 45 s were
run. Finally the specimens were kept at 72 °C for 10 min. Amplified fragments were detected by time-resolved fluorometry as previously described. 23

Results

The theoretically calculated sensitivity of the PCR–TRF was one to two Chlamydia organisms and the PCR–TRF showed positive results in a further two four-fold serially dilution steps as compared with culture. None of the tested organisms showed a positive result.

The arbitrary loss of DNA during the extraction procedures (the mean relative reduction in PCR–TRF-signal) for the various investigated extraction procedures were the following: G-NOME DNA (99%), QIAmp Kit (99%), guanidinium thiocyanite (79%) phenol/chloroform extraction (78%), Chelex Resin (70%), proteinase K treatment (0%), Amplicor HCV specimen preparation kit (0%). The optimal DNA extraction procedures with regard to preservation of DNA were thus proteinase K treatment and the Amplicor HCV specimen preparation kit. However, none of these methods gave a positive result when the 10^3 Chlamydia organisms were added to the clinical samples, and the two tests could therefore not sufficiently remove inhibitors in clinical samples. Taking into account both the loss of DNA during extraction and the ability to remove inhibitors, the phenol/chloroform extraction was the preferred extraction procedure.

A total of 124 samples from the 20 patients were prepared including four cases of mural thrombus. The number of samples from each patient was two to 10. Of the 124 samples, inhibition to PCR was found in 34 specimens from 11 patients. Four of the specimens showing inhibition were in the mural thrombus, 22 in the media samples, the remaining in intima specimens, but all patients had their intima examined without inhibition, while the examination of media in four patients occurred with inhibition. Neither in these specimens with inhibition nor in the remaining 90 samples could C. pneumoniae DNA be found (95% CI: 0.00–16.8%).

Discussion

Despite use of a sensitive inhibitor-controlled PCR–TRF for detection of C. pneumoniae, we were not able to demonstrate this organism in a Danish population with symptomatic AAA. This is in contrast to the data by Ong et al. 18 who recently found C. pneumoniae DNA in 11 of 25 aortic atherosclerotic lesions (44%). Campbell et al. 13 detected C. pneumoniae in 32% of 38 samples, and Kuo et al. 14 detected C. pneumoniae in 20 of 36 specimens (56%), whereas Weiss et al. 17 only detected one PCR-positive case among 58 specimens from 50 patients using the same methods. Atherosclerosis and AAA, although associated, may not be identical diseases, and this might explain why we were unable to detect C. pneumoniae in AAA-walls. More than 50% of AAA found by screening are in patients without a previous medical record of atherosclerotic disease. 29 Furthermore, atherosclerosis seems not to correlate to the size of AAA. 26,27

Another explanation for the diverging results could be different prevalences of C. pneumoniae in different countries. No such evidence currently exists. It may be that our test system has a lower sensitivity compared with the method applied by Ong et al. 18 However, the DNA extraction method used in the two studies was nearly identical, and the sensitivity of our PCR–TRF is near optimal. Furthermore, the applied extraction procedure and PCR–TRF have been successfully applied with positive findings on middle ear secretions from children suffering from otitis media. 29 Therefore it is unlikely that methodological differences can explain the diverging results.

In conclusion, unlike studies of atherosclerotic lesions, we were not able to detect C. pneumoniae in symptomatic AAA, suggesting that AAA and atherosclerosis are different disease entities.

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References


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