Detection of *Chlamydia pneumoniae* (*Chlamydophila pneumoniae*) in endarterectomy specimens of coronary heart diseases patients

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**Background & objectives:** Association of *Chlamydia pneumoniae* with atherosclerosis and coronary artery disease is debated. Increased antibody levels against *C. pneumoniae* in patients with coronary artery disease is widely reported. Direct evidence would be demonstration of *C. pneumoniae*, its antigen or genome in the diseased arterial tissue. This study was thus conducted to look for antigen or genome of *C. pneumoniae* in coronary artery specimens from patients with coronary artery disease along with serology.

**Methods:** Sixty two end arteriotomy specimens of discarded coronary arteries from patients of coronary heart disease were tested for presence of *C. pneumoniae* genome using 2 nested PCR assays and antigen detection by immuno-fluorescence assay. Presence of species specific antibodies were also tested in the patients.

**Results:** *C. pneumoniae* could not be detected by PCR or immunofluorescence assay in any specimen. *C. pneumoniae* Ig G antibody was detected in 42 of the 62 (67.7%) patients studied, compared to 10 of the 23 (43.47%) of controls. Moreover 18 of 62 (29%) patients compared to 4 of 23 (17.39%) controls possessed IgA antibodies.

**Interpretation & conclusions:** Association of *C. pneumoniae* and coronary artery disease would not be established by genome or antigen detection. However, *C. pneumoniae* antibodies were detected in more number of patients than controls. More studies are required to reach to a conclusion.

**Key words** Atherosclerosis - *Chlamydia pneumoniae* - coronary artery disease - End-arteriotomy - immunofluorescence assay - nested polymerase chain reaction (PCR)

*Chlamydia pneumoniae* (now renamed *Chlamydophila pneumoniae*) was detected in coronary atheromatous plaques in early 1990s suggesting an aetiological association. Subsequently, studies were conducted in different countries to prove association, yielding divergent results. Some were based on antigen detection or polymerase chain reaction assays demonstrating *C. pneumoniae in situ* in the atherosclerotic plaques. However, a majority were based on *C. pneumoniae* antibody detection. Atherosclerotic plaques may harbour the organisms, but an exclusive causal relationship between *C. pneumoniae* and coronary heart disease is yet to be proven.

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Such studies are sparse from Asian population\textsuperscript{9}. In India, a few association studies conducted in past were of seroepidemiological in nature\textsuperscript{10}, showing increased prevalence of \textit{C. pneumoniae} antibodies in patients with coronary heart disease\textsuperscript{11}. However, to suggest disease association, direct demonstration of the organism (\textit{C. pneumoniae}), its antigen or genome in the diseased arterial tissue is essential.

The present study was conducted to look for \textit{C. pneumoniae} antigen and genome by immunofluorescence assay and PCR assay in end-arteriotomy specimens from patients with coronary heart disease undergoing coronary artery bypass grafting (CABG) procedure. \textit{C. pneumoniae} species-specific IgG and IgA antibodies were also detected in these patients and controls by microimmunofluorescence assay.

**Material & Methods**

**Specimen collection:** Sixty two specimens of discarded coronary arteries were collected from patients undergoing CABG procedure at the Department of Cardiovascular and Thoracic surgery of All India Institute of Medical Sciences, New Delhi after written consent, from May 2000 to January 2003. All patients were males between 45-75 yr (mean age 55.12 yr SD ± 12.5 yr).

End-arteriotomy specimens of coronary artery were collected in 0.2 M sucrose phosphate buffer and homogenized thoroughly. Small antigen dots were made in the wells of teflon coated slides and rest was preserved at -70°C for PCR assay.

After written consent 5 ml of blood was collected from each patient and 23 age matched controls (from patients attending Cardiology Department for reasons other than atherosclerosis and CABG procedure.)

**Antigen detection:** \textit{C. pneumoniae} antigen was detected by indirect immunofluorescence assay using \textit{C. pneumoniae} monoclonal antibodies (DAKO diagnostic Ltd, Denmark) and fluorescein isothiocyanate tagged anti mouse IgG (1:30) (DAKO, Denmark) as second antibody\textsuperscript{12}.

**PCR assay for detection of \textit{C. pneumoniae} DNA**

DNA was extracted from the coronary artery specimen using SDS (Sigma, USA) and proteinase K (Life Technology Inc., USA) treatment and standard phenol chloroform extraction and ethanol precipitation procedure\textsuperscript{13}.

**Primers and PCR conditions:** Two sets of primers were synthesized on an Applied Biosystems DNA Synthesizer (Model 392B, USA). Two separate regions of \textit{C pneumoniae} genome were amplified using nested PCR assays\textsuperscript{8,14}. The sequences for amplification of 437bp Pst1 fragments of \textit{C. pneumoniae} were; Forward: 5’ GTT GTT CAT GAA GGC CTA CT3’ and Reverse: 5’ TGC ATA ACC TAC GGT GTG TT3’\textsuperscript{19}.

A 190bp fragment within the earlier Pst1 fragments was amplified using primers; Forward: 5’ TTG AGC ATA TTC GTG AGG 3’, Reverse: 5’ GTA CAG TTT CTG CGT TAG 3’\textsuperscript{14}. Primers were synthesized in the laboratory on a DNA/RNA synthesizer (Applied Biosystems, USA).

Nest PCR\textsuperscript{15} assay was repeated for amplification of 333bp region of \textit{C. pneumoniae} using another set of primer and positive control (Genomic DNA of \textit{C. pneumoniae} obtained from Prof. J Schatcher, Chlamydia Research Laboratory, University of California, USA)\textsuperscript{16}.

The sequences of primers were: CP1 (sense): 5’ TTA CAA GCC TTG CCT GTA GG 3’, CP2 (antisense): 5’ GCG ATC CCA AAT GTT TAA GCC 3’. Sterile Mili-Q water was used as negative controls.

A 207 bp region of \textit{C. pneumoniae} within the 333 bp region were amplified using primers: CPC (sense): 5’ TTA TTA ATT GAT GGT ACA ATA 3’, CPD (antisense): 5’ ATC TAC GGC AGT AGT ATA GTT 3’.

The PCR products were electrophoresed on 0.8 and 2 per cent agarose (Sigma, USA) gels and visualized under gel documentation system. (Syngene, USA).

**Sequencing of the PCR positives:** The PCR positive results were verified using sequencing of the products. Briefly, the PCR positive DNA bands from agarose gels were excised with a sterile scalpel blade, DNA was purified from the gel using Quiagene gel purification kit according to manufacturer’s instructions. (Quiagene, USA). The purified DNA was sequenced manually using Sanger’s dideoxy chain termination method\textsuperscript{17} using T7 DNA polymerase (Sequence version 2.0, USB, USA).

**Antibody detection:** Microimmunofluorescence assay was used for detection of species specific \textit{C. pneumoniae} Ig G and Ig A antibodies in patients sera along with 23 controls using Multiscreen Chlamydia test kit (IO International, UK) as described before\textsuperscript{11}.

**Statistical analysis:** Serology data were analysed using chi square test.

**Results**

**Antigen detection:** None of the specimens tested for \textit{C. pneumoniae} antigen by immunofluorescence assay gave positive results.
PCR assays: In the PCR assay for amplification of 437bp Pst I fragment, 6 specimens gave positive results (Fig. 1), in the touch down PCR assay for amplification of 190 bp fragment, 3 of the 6 gave positive results. However, along with the band of 190bp another band of slightly larger size approx. 300bp was observed in 2 of these (Fig. 2).

In sequencing, the sequence obtained for 190 bp fragment were: GGGAACTTGATTGAGCACATAC/TCGTGAGGGAGATTGATCGTATGTTATGTTT
TCCAATTGTTTTTCCTCCAGGGAGTTGTTTGCC
TAGAGCATCTAAAGACAGTG
ATGTTCTGGTAAACACATAGTGGAAATTCTATC
TCTAGGGTCTTCAGACTAAC
GGAGAAAATCTGAATCATCGAATTGCGG
CGCCTCCGACG

However, the published sequence for C. pneumoniae for Pst I restriction fragment of C. pneumoniae was:
CCATTATCCACCGTCTACAGCAGAAATCTGTT
GTTCATGAAGGCCTACTCTTT
GATCAAGAGCAAATAGAAACAGGATAAGAAAG
AAGATTAGTGATCTTTTTAA
TGCCCTACTGTAATAGTGATTTGAAA
GGACTTATCTAGATTACGA
AAGCGGATTAACAGCTAGAAATCAATTATA
AGACTGAAGTTGAGCATACT
CGTGAGGGAGATCGAGATTTAGATCATGTGTT
CATTCGGCAAGGCTAAAGTC
TACGTGGCTCTAAGAGAAAGCTTCAAGTTGG
AGATAATGGCTGGACGACA
CGGAAATACCAAGCTGTTTTGACAATATGGTTC
GGACTTCTATCAGATTACGA
CTCTCTAACGGGAAATCTGAATCATCGAATTGCGG
CGCCTCCGACG

Only 29 per cent homology was observed between the sequence obtained and the published sequence for C. pneumoniae.

In PCR for amplification of 333 bp segment of C. pneumoniae DNA, none of the coronary artery specimens gave positive results although good amplification was noted in positive control. (Fig. 3).

Antibody detection: IgG antibodies against C. pneumoniae was detected in 42 of the 62 (67.7%) coronary artery disease patients and 10 (43.4%) of the 23 controls. The control specimens had low antibody titre (1:64 in 4 and 1:16 in 6) while in coronary artery patients antibody titres were higher (1:128 in 17 and 1:64 in 22) (P<0.05).
IgA antibodies were detected in 18 (1:8 in 10 and 1:4 in 8) of the 62 (29%) coronary artery disease patients with the higher titre and 4 of 23 (1:8 in 2 and 1:4 in 2) (17.3%) controls.

**Discussion**

After the initial hypothesis of association between *C. pneumoniae* and coronary heart disease (CHD) by Saikku et al. many such studies have been published, using different diagnostic criteria like electron microscopy, immunocytochemistry, direct immunofluorescence and PCR assay demonstrating *C. pneumoniae* in-situ in the atherosclerotic plaques.

In the present study, we used highly specific nested PCR assays along with direct antigen and antibody detection by immunofluorescence to study the association. Although, we could not detect *C. pneumoniae* specific gene by PCR assay or *C. pneumoniae* antigen by immunofluorescence, serologically higher titres of IgG were observed in CHD patients than in control subjects. *C. pneumoniae* IgM antibodies were reported to have no diagnostic value in coronary heart disease, hence were not assessed in this study.

It has been suggested that methodological problems may account for the inability to detect *C. pneumoniae* in coronary artery specimens. Polymerase chain reaction assay technique needs a proper care in conducting the tests, as common problems include nonspecific background DNA products, primer dimer formation and mutation or heterogeneity due to misincorporation of nucleotide in the genome. In this study, in the primary PCR round we got 6 positive results but in the touch down PCR assay, only 3 gave positive results. In the absence of culture confirmation, we did nucleotide sequencing of the 3 amplified fragments. But only 29 per cent homology was observed with available sequences forcing us to conclude non specific amplification.

The nested PCR assay was further repeated to amplify a separate region of *C. pneumoniae* genome, but no positive results were noted. The reported rate of detection of *C. pneumoniae* DNA within atherosclerotic lesions by different PCR technologies in different countries varies between 0 to 100 per cent. It is possible that the clinical specimens analyzed contained almost no *C. pneumoniae* DNA, that the occasional positive results were from incidental contamination, and that the few methods yielding high proportion of positive results had an inherent yet undetected error. However, it is also possible that *C. pneumoniae* DNA was present in the majority of samples and that the few methods yielding positive results were closer to the truth, while those failing to yield a positive reaction could not detect the target either due to the failure of DNA extraction from atheroma tissues or due to some undetected inhibitory mechanism. The size and composition of atherosclerotic lesion may also affect the rate of detection of *C. pneumoniae*. In this study, we had used end arterectomy specimens enabling us to use the entire atheroma present to be studied, eliminating scanty specimen availability as the cause for non detection.

Furthermore, discrepant results probably also reflect characteristics of the study population. It is unknown which factors favour the colonization of atherosclerotic lesions by *C. pneumoniae*. The cardiovascular risk profile of our patient population was not significantly different from the study population from other countries, but more studies need to be conducted to determine the sensitivity and specificity of PCR by testing a large number of clinical specimens from asymptomatic individuals.

In our study, though *C. pneumoniae* Ig G and IgA antibodies have shown higher positivity in CHD patients with higher titre than in control, it seems unlikely that serology alone will identify individuals at high risk for CHD.

In conclusion, our findings from a limited number of samples do not support the hypothesis that *C. pneumoniae* plays a major role in coronary artery disease. Further studies need to be done on a larger sample with more specific techniques to confirm the findings.
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References


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