Detection of *Chlamydia pneumoniae* in atherosclerotic tissue: a comparative study of PCR and immunocytochemistry

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ABSTRACT

The reported prevalence of *Chlamydia pneumoniae* in atherosclerotic tissue appears to depend on the detection system used. This introduces problems in determining the role of *C. pneumoniae* in atherosclerosis. This study analyses the sensitivity and performance of molecular diagnostic methods for the detection of *C. pneumoniae* and polymerase chain reaction (PCR) inhibitors in atheromatous tissue. Atherosclerotic tissue taken from 30 coronary endarterectomies, nine coronary arteries from explanted hearts, 16 carotid and two femoral endarterectomies are studied. Nested PCR (nPCR) assays targeting the *PstI* restriction fragment, the *OmpA* gene and the CRP operon of the chlamydial genome and immunocytochemistry (ICC) are used. Internal controls (IC) are constructed to co-amplify with the specific amplicons and identify the presence of inhibitor. The *OmpA*, *PstI* and CRP operon PCR assays had similar analytical sensitivities. However, the *OmpA* PCR was most affected by PCR inhibitors. Despite this, eight samples (14%) tested positive in the *OmpA* nPCR and no positives were found using the *PstI* or CRP operon nPCRs. Primary isolates of *C. pneumoniae* obtained from 12 patients with acute respiratory infection were positive in all three assays. Of the 48 specimens available for ICC, 33 (69%) were positive for chlamydial antigens. These included samples found positive by PCR. Dilution of samples to eliminate PCR inhibitors may have contributed to the discordant ICC and PCR results. The *OmpA* PCR, when used with an IC to identify samples with PCR inhibitors, is a reliable tool. However, the sensitivity of the ICC methods justifies their continued use.

KEY WORDS: Atherosclerosis. Chlamydia pneumoniae. Immunocytochemistry. Infection. Polymerase chain reaction.

Introduction

Since the discovery of *Chlamydia pneumoniae* in atherosclerotic tissue 13 years ago,[1] the organism and its relationship with coronary artery disease (CAD) have been scrutinised intensely. Based on serological findings as well as molecular and immunocytochemical (ICC) association studies, treatment objectives have been set and large-scale studies initiated. However, the success story is tainted with a complete lack of agreement on the proportion of individuals carrying the infectious agent. Whether atheromatous plaque tissue or circulating leucocytes are tested, some have found no *C. pneumoniae* at all,[2–5] while others report high infection rates.[6–8]

The most commonly used *C. pneumoniae* detection methods are the polymerase chain reaction (PCR) and ICC. Discrepancies exist between ICC data and PCR findings, with the latter showing a lower detection rate than ICC despite having the higher sensitivity.[9] Although comparisons of ICC and PCR, using infected murine tissue, have shown good agreement between the tests,[10] this has not been the case with human samples.[9] These lower detection rates in man have been attributed to the presence of PCR inhibitors. Whether or not such inhibitors have an effect on detection sensitivities is not known.

The contribution that the choice of amplification primers makes to variations in detection rates has been studied by Mahony *et al.*,[11] who found that *OmpA* PCR was able to identify a higher proportion of positive peripheral blood leucocytes than did other commonly used PCRs.[11] This could not be attributed to the analytical sensitivity of the assays, as all showed similar sensitivities.

The objective of the present study is to construct internal controls (IC) to be used in PCR techniques that target three separate regions of the *C. pneumoniae* genome. These ICs, capable of amplifying alongside the target sequences, establish not only assay sensitivities but also identify samples with PCR inhibitors. The work is carried out on atherosclerotic plaque tissue.

The potential significance of the presence of *C. pneumoniae* in atherosclerotic tissue and its role in CAD justifies an investigation into the causes of discrepant reports in the prevalence of this organism in atherosclerotic arteries. Accurate diagnosis of infection is crucial if treatment is to be of value.

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Materials and methods

Arterial samples

Arterial samples were obtained from 57 patients (47 men, 10 women; mean age [SD] 60.4 [11.0] years). In 30 patients (27 men, three women; mean age [SD] 61.6 [7.9] years) samples were obtained by endarterectomy during elective or urgent coronary artery bypass surgery (CABG).

A further nine coronary arteries were obtained from the explanted hearts of patients undergoing cardiac transplantation (eight men, one woman; mean age [SD] 44.3 [11.5] years). Endarterectomy material obtained from 16 carotid and two femoral arteries was also included (12 men, four women; mean age [SD] 65 [9.9] years). Material to be used in the PCR methods was frozen at –70˚C immediately following removal.

Broncoalveolar lavage samples, taken from 12 patients with respiratory disease, were cultured and proved positive for *C. pneumoniae*. Extracted DNA from these cell cultures was used as positive controls.

Immmunocytochemistry

Cryostat sections (5 µm) of atherosclerotic tissue were treated with rabbit serum (DakoCytomation) containing four drops/mL avidin solution (avidin/biotin blocking kit, Vector Laboratories), washed with phosphate-buffered saline (PBS) and incubated with mouse anti-*C. pneumoniae*-specific antibody containing four drops of biotin solution (clone RR402, DakoCytomation). Mouse IgG3 (Sigma) was used as a negative control. After overnight incubation at 4˚C, the sections were washed and incubated with biotin-conjugated rabbit anti-mouse immunoglobulins (DakoCytomation), followed by streptavidin/FITC (DakoCytomation). Immunostaining was examined microscopically.

Polymerase chain reaction

The arterial samples were digested with proteinase K and subjected to phenol-chloroform DNA extraction. Nested PCR for amplification of three separate regions of the chlamydial DNA (the *PstI* restriction fragment,[12] the *OmpA* gene[13] and the CRP operon [nucleotide positions of first- and second-round amplimers: 642206-643240 and 643094-642677; accession number AE001363]) were performed in the presence and absence of IC, designed to co-amplify with the specific amplimers in order to establish the sensitivity of the assays and identify samples inhibitory to PCR.

The amount of IC to be added to the PCRs was predetermined to be sufficiently low such that amplification would be affected by the presence of inhibitors. The sequence of primers used for amplification, the size of the amplimers, the reaction conditions and copy number of IC used are shown in Table 1.

First-round amplification reactions were performed using 500 ng DNA in 25 µL reaction volumes. One microlitre of the product was used subsequently in nPCR reactions. Samples shown to contain inhibitors were diluted 10- and 100-fold and retested. DNA extracted from Hep2 cells infected with the TW-183 strain of *C. pneumoniae*, as well as clinical isolates of *C. pneumoniae* obtained from 12 cases of acute respiratory infection were used as positive controls.

Construction of internal controls

To construct the ICs for the *PstI* PCR, a 261-bp fragment of the pUC18 vector corresponding to positions 1720-1980 was amplified using hybrid primers carrying sequences complementary to both pUC18 and *PstI*. The sequence of the primers was as follows: 5'-**GTGTCATTCGCCAAGGTTAA** TACGGGAGGGCTTACCA and 5'**ACCTGTCCAAGGTTCAT CCT**GCCATACCAAACGACGAG. The bold regions are identical to the primers used in the amplification of the *PstI* in the nPCR reactions (Table 1). The PCR products, now 301 bp in fragment size, were re-amplified using a second set of hybrid primers of the following sequence: 5'-GTTGT TCATG AAGGCCTAGTGTCATTCGCCAAGGTTAA and 5'-TGCATAA CCTACGGTGTGTTACCTGTCCAAGGTTCATCCT, comprising the first- and second-round amplification primers.

The purpose of using hybrid primers was to generate a construct that would later be used in nPCR reactions co-amplifying with the specific amplicon. The products, now 339 bp in size, were gel-purified and cloned into pCR2.1 T-A cloning vector (Invitrogen). Purified plasmid DNA was quantified and the copy number determined.

The IC for the *OmpA* PCR was constructed as described for *PstI*. A 105-bp region of the pUC18 vector corresponding to positions 202-306 was amplified using hybrid primers carrying sequences complementary to both pUC18 and *OmpA*. The sequence of the primers was as follows: 5'-**TTATTAATTGATGGTACAATA**ACGCACAGATGCGTA AGGAG and 5'-**ATCTACGGCAGTAGTATAGTT**GCGTAA TAGCGAAGAGGC. The bold regions are identical to the primers used in the amplification of the *OmpA* in the nPCR reactions (Table 1). The PCR products, now 147 bp in size, were re-amplified using a second set of hybrid primers of the following sequence: 5'-TTATTAATTGATGGTACAATATTACA AGCC TTGCCTGTAGG and 5'-ATCTACGGCAGTAGTA TAGTT GCGATCCCAAATGTTTAAGGC, comprising the first- and second-round amplification primers. The 188-bp product was cloned as described above.

The IC for the CRP operon consisted of the same chlamydial genomic DNA but carried a deletion of 232 bp between the nested primer annealing sites. The shorter fragment size distinguished the control from the positive product. The deletion was introduced by *BfaI* (New England Biolabs) digestion of gel-purified 1034 bp first-round amplification product. The digestion yielded 497 bp and 534 bp bands. A separate sample was digested with *AseI* (New England Biolabs), yielding 265 bp and 769 bp products. The 534-bp and 265-bp fragments were ligated together, cloned into a pCR 2.1 vector and propagated in *Escherichia coli* strain INVαF, using a T-A cloning kit (Invitrogen).

Prior to use, the optimum amounts of IC to be added to the amplification reactions were determined. This was carried out by titrating the ICs in the presence and absence of *C. pneumoniae* DNA. The lowest IC copy number that consistently amplified in the presence of cellular DNA was chosen for routine use (Table 1).

Myoglobin PCR

Amplification of the myoglobin gene was used to confirm integrity of the extracted DNA.[14] The primers and reaction conditions used are shown in Table 1.

Results

There were marked differences in the performance of the

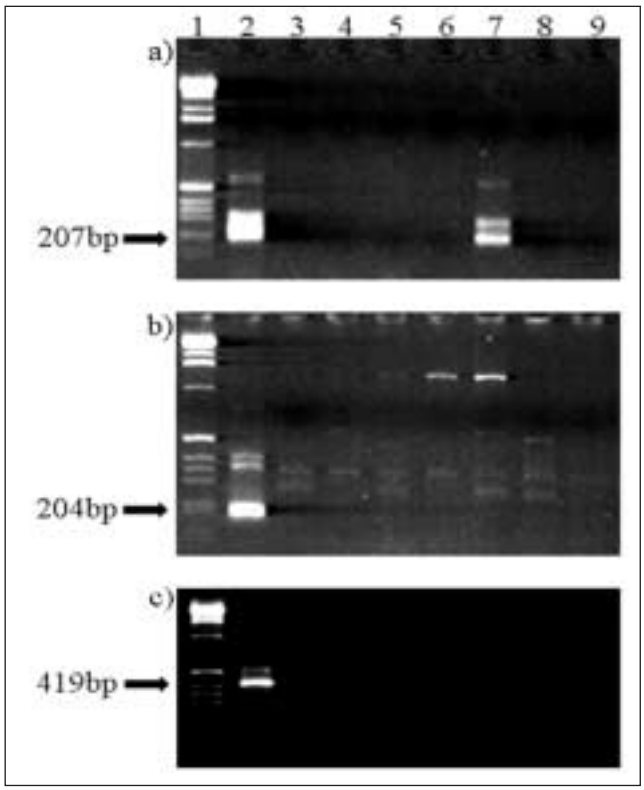

Fig. 1. Nested PCR analysis of *C. pneumoniae* genomic DNA in atherosclerotic plaques. Lane 1 represents the molecular weight marker. Lane 2 shows positive control DNA. Lanes 3–8 show PCR results from DNA extracted from atherosclerotic material. Panel a) shows results using the *OmpA* nPCR assay, sample 7 is positive. Panel b) shows results using the *PstI* assay and panel c) the CRP operon assay, both of which resulted in negative findings.

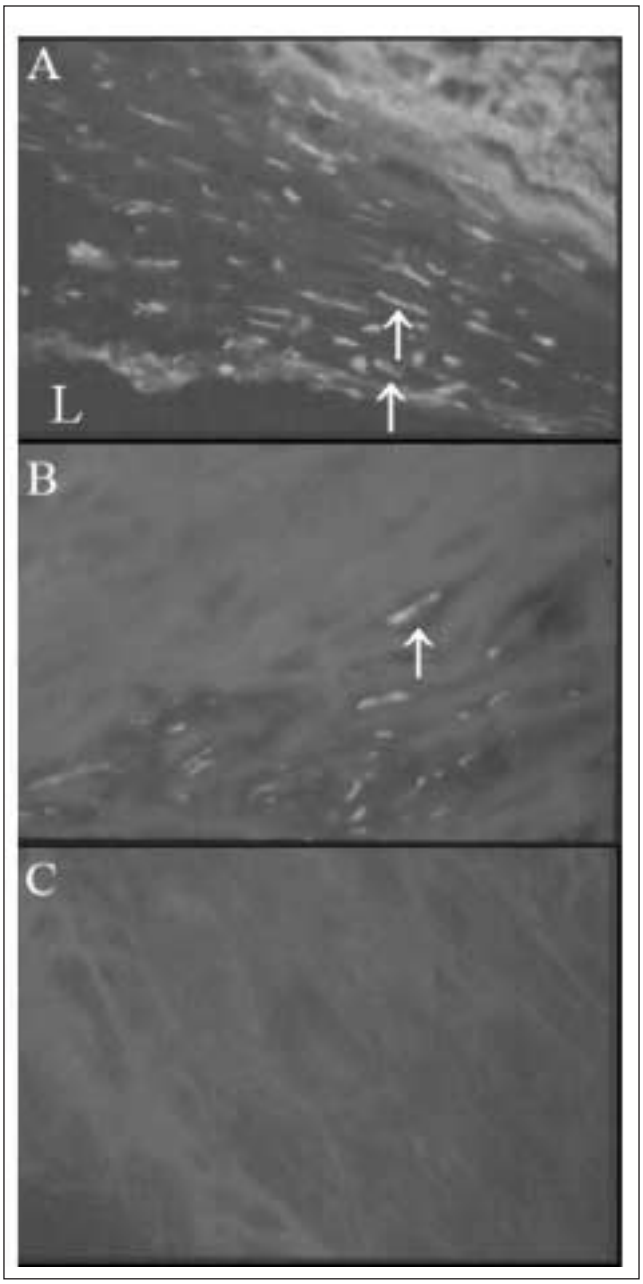

Fig. 2. Immunocytochemical staining of *C. pneumoniae* in 5-µm cryostat sections of coronary atherosclerotic lesions. Panels A–C show the intimal areas from three patients. *C. pneumoniae*-positive cells are indicated by arrows in panels A and B. Panel C shows an area of a section containing no *C. pneumoniae*. L indicates the lumen of the artery (original magnification x400).

three nPCR assays in their abilities to detect *C. pneumoniae* DNA in atheromatous plaque tissue. While the *OmpA* nPCR in the absence of IC identified eight out of 57 specimens (14%) as carriers of *C. pneumoniae*, the *PstI* and CRP operon reactions failed to detect any positives. Representative results are shown in Figure 1. Using the *OmpA* nPCR, the prevalence of *C. pneumoniae* DNA in the coronary endarterectomy samples was three out of 30 (10%) compared to two out of nine (22%) in the explanted arteries and three out of 18 (16.7%) in femoral and carotid endarterectomies. The myoglobin PCR suggested that the integrity of the DNA in all samples was intact. The failure of the *PstI* and CRP operon PCRs to identify positive samples could not be attributed to lower assay sensitivities.

All three nPCR procedures identified laboratory cultured clinical isolates of *C. pneumoniae* obtained from 12 patients with respiratory tract infections (data not shown). When the sensitivities of the *PstI* and the *OmpA* nPCRs were compared with the CRP operon assay, using DNA extracted from cultured organism, the CRP nPCR proved to be 10-fold more sensitive (data not shown). Despite this the only positive reactions obtained in atheromatous tissue were in the *OmpA* nPCR.

To investigate further the issue of assay sensitivity and detect PCR inhibitors, the nPCRs were performed in the presence of ICs. The constructs co-amplified alongside the specific amplicons but were of different length, which allowed them to be distinguished from the positive product. Serial dilutions of the constructs alone demonstrated that all three nPCR assays were able to detect between one and 10 copies of their target amplicons (data not shown). In the presence of chlamydial DNA or DNA from clinical samples, however, the three procedures had sensitivities varying from one to 10 copies (CRP) to 100 copies (*PstI*) and 1000 copies (*OmpA*).

Introduction of the IC into each reaction allowed any inhibitors present in the samples to be identified. Although no problems were encountered in the amplification of the

Table 1. Primer sequences and reaction conditions used in the PCR amplifications.

Target	Primers	Product size	No of cycles	$MgCl_2$ (mmol/L)	Anneal temp (˚C)	IC copy number
PstI	First round primers	438	40	1.5	55	100
	HL1 5'-GTTGTTCATGAAGGCCTA					
	HR1 5'-TGCATAACCTACGGTGTGTT					
	Nested primers					
	HM1 5'-GTGTCATTCGCCAAGGTTAA	204	30	1.5	43	
	HR2 5'-ACCTGTCCAAGGTTCATCCT					
OmpA	First round primers	333	40	1.5	Stepdown	1000
	CP1 5'-TTACAAGCCTTGCCTGTAGG				65–55	
	CP2 5'-GCGATCCCAAATGTTTAAGGC					
	Nested primers	207	30	1.5	50	
	CPC 5'-TTATTAATTGATGGTACAATA					
	CPD 5'-ATCTACGGCAGTAGTATAGTT					
CRP operon	First round primers	1034	35	2.0	60	1–10
	CPOF 5'-TGCCGCATTGTAGATTGTTG					
	CPOR 5'- CTGGTTGACCGCATTTAGTA					
	Nested primers	417	25	3.0	62	
	CPIF 5'-ACAGAGTGTAACTCTCAAGC					
	CPIR 5'-TTCTACGGACAAGTCTAACC					
Myoglobin	5'-AACATGACAGGTCCTCTTGG	439	35	1.5	55	
	5'-CATGGCGCAGTCTGAA					

myoglobin housekeeping gene, in the case of the CRP operon PCR the IC did not amplify in 13/57 (31%) samples. These samples required retesting after a 10-fold dilution. Amplification of the IC in the *PstI* and *OmpA* PCR protocols were more severely affected by the presence of inhibitors, as samples required retesting at 100-fold dilution before amplification of the IC was observed. When using the *OmpA* IC PCR, seven of the eight positive samples required 100-fold dilution before the IC was amplified successfully.

The benefit of introducing ICs to identify PCR inhibitors was counteracted by the competitive nature of these constructs. As the primer recognition sites were shared between the ICs and the specific amplicons, reaction sensitivities were compromised. Samples found positive in the absence of IC were negative when the construct was introduced into the reaction mixture. All samples therefore had to be tested with and without IC to avoid false-negative results. When dilution of a sample was necessary (i.e., when the IC did not amplify), suggesting the presence of inhibitors in the reaction mix, the diluted samples were tested in the presence and absence of ICs.

Forty-eight atheromatous plaque samples were available for testing by ICC. Of these, 33 (68.7%) were found to carry chlamydial antigens. Six of the eight samples positive by PCR were available for analysis by ICC. All six were found to have chlamydial proteins. Characteristic ICC staining from three different patient samples is shown in Figure 2.

Discussion

This study compared three nPCR assays and found *C. pneumoniae* DNA in eight out of 57 (14%) arteries using an *OmpA* nPCR. In contrast, both the *PstI*-based and the CRP operon-based assays failed to detect any positives, despite comparable sensitivity to the *OmpA* nPCR when used on *C. pneumoniae*-infected clinical samples and the ability to detect lower copy numbers of the IC under laboratory conditions. Thus, sensitivity of the assay does not predict its ability to detect DNA of the organism in atheromatous plaque tissue. The findings are in agreement with the report by Mahoney *et al*.,[11] who found that although the analytical sensitivity of the *OmpA* PCR on clinical isolates was comparable to the sensitivity of four other target-region PCR assays, including the *PstI* PCR, the *OmpA* region was a more appropriate target for amplification of *C. pneumoniae* in leucocytes.

The *PstI*, 16SRNA and *OmpA* primers are used widely. However, studies using *OmpA* primer targets have slightly higher detection rates than *PstI* or 16SRNA primer targets.[3,4,6,7,8,15–17] The current study's findings and those of Mahoney *et al*.[11] suggest that the widespread use of this region for amplification is justified. The choice of the target region, however, is unlikely to be the only reason for the reported variation in *C. pneumoniae* carriage rate.

Multicentre studies using *OmpA* PCR on clinical and laboratory isolates of *C. pneumoniae* have shown up to 40% difference in the detection rates of the organism. When laboratories used identical primers in the *OmpA* target region, inter-laboratory variations were still apparent. The differences were not simply between laboratories. Within-centre agreements of in-house PCRs varied (Kappa=0.19 to 0.74) on tests carried out one month apart.[18]

What gives an advantage to one set of primers over another may be the size of the PCR amplicon. Larger fragments may be more prone to degradation in tissue and be more troublesome to amplify. The fragments amplified in

most published studies are of approximately equal length (500 bp), the exception being a study by Blasi *et al*.,[6] where 51% of samples were found to be positive, amplifying a 1292 bp region.[6] This suggests that the relatively longer first-round product had little effect on the reaction. In the present study, the *OmpA* first-round amplicon size was 333 bp, compared to 438 bp for the *PstI* primer and 1034 bp for the CRP operon primer. Although it is possible that the long CRP operon target may be degraded in some samples, the negative results obtained in the *PstI* reaction cannot be explained on the basis of fragment size alone.

Strain variation in the *C. pneumoniae* genome that gives advantage to one set of primers over another may offer an explanation for differential performance of primer sets. The PCRs conducted in the present study were evaluated on 12 cultured clinical isolates and all three assays amplified *C. pneumoniae* sequences successfully. Thus, the likelihood that nucleotide variation affects primer binding sites in atheromatous plaque tissue is small.

A problem, which may be of particular consequence when testing atherosclerotic vessels, is the presence of PCR inhibitors. Wong *et al*.[8] found that atherosclerotic vessels are more likely to contain PCR inhibitors than non-atherosclerotic vessels, and Thomas *et al*.[15] showed that tissues with severe lesions tend to contain more inhibitors than tissues with mild lesions, with lipid and calcium being the main sources of inhibition.

The significance of PCR inhibitors and the mechanisms by which they interfere with amplification have been reviewed elsewhere.[19] In view of this, the important features of the present study were construction of ICs able to detect PCR inhibitors and the use of quantified DNA, the integrity of which had been tested by the amplification of the housekeeping gene. Discordant studies in the literature may reflect absence of such controls. DNA quantification and the assessment of integrity are essential if comparisons are to be made not only between different studies but also in individual studies attempting to compare healthy tissue with diseased samples.

However, assay variations reported here are unlikely to be solely a reflection of the inhibitory substances present in tissue. The *OmpA* IC construct, when amplified in the presence of human DNA, was the one most adversely affected by inhibitors, requiring 1000 copies for detection. Yet *OmpA* was the target amplified successfully.

The strategy used in this study to evaluate PCR methods does not fully account for all variations in the PCR. As shown in the results section, introducing an internal control reduces efficacy of the diagnostic amplification procedure. To establish that both diagnostic and internal PCRs are equally effective requires the use of real-time PCR. Most laboratories engaged in PCR-based identification of bacterial infection will not have access to real-time PCR equipment. This would need to be addressed if accurate diagnosis using tissue samples prone to contamination with reaction inhibitors is required.

As reported by others, ICC generated a larger proportion of positive results. All samples found containing *C. pneumoniae* DNA by PCR and available for testing by ICC were also found to be positive by this method. Therefore, ICC served to confirm the positive PCR data. However, 27 samples had detectable bacterial proteins in the absence of DNA. Dilution and retesting of these samples using all three primer sets failed to identify bacterial nucleic acids in these tissues. Although dilution of samples eliminates the inhibitors, low copy numbers of *C. pneumoniae* may be responsible for subsequent failure of the amplification reaction. An alternative explanation is that although chlamydial genomic DNA may be easily degraded, chlamydial membrane proteins may survive within the plaque.[20]

In conclusion, the present study identified an important source of discrepancy in the detection of *C. pneumoniae* DNA in atherosclerotic tissue. It showed that the PCR primer sequences used, in addition to the presence of PCR inhibitors, might be responsible for the variation in positive findings. In view of the proposed association between *C. pneumoniae* infection and atherosclerosis, there is a requirement for appropriate diagnostic strategies for identification of carriers. Without reproducible techniques, however, it is difficult to evaluate the benefits of antibacterial treatments. □

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