

# Simplified preparation of human arterial sections for PCR analysis of *Chlamydia pneumoniae* and human DNA

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## Abstract

**Aims**—To investigate multiple techniques for the preparation of solid tissue for polymerase chain reaction (PCR) analysis, and to identify the most simple techniques for routine use in the laboratory.

**Methods**—Techniques for the preparation of arterial tissue samples including homogenisation, ultrafiltration, and treatments involving proteinase K, Gene Clean™, lectin, and Fe<sup>3+</sup> specific chelators were evaluated using the PCR to amplify both *Chlamydia pneumoniae* and human DNA.

**Results**—Treatment with either Gene-Clean or lectin and the Fe<sup>3+</sup> specific chelator deferoxamine mesylate removed PCR inhibitors from tissue homogenates. Homogenisation followed by GeneClean treatment resulted in the amplification of *C pneumoniae* DNA from within a section of atherosclerotic carotid artery, implying that *C pneumoniae* elementary bodies had been disrupted. In eight further clinical samples from patients not known to have *C pneumoniae* infection, human DNA was amplified and no cross contamination was observed between samples. These samples contained no evidence of *C pneumoniae* by PCR.

**Conclusions**—A simple preparation of solid tissue for PCR analysis, involving homogenisation followed by GeneClean treatment has been developed, and is effective for the amplification of both *C pneumoniae* and human DNA.

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**Keywords:** polymerase chain reaction; sample preparation; *Chlamydia pneumoniae*

Four species of *Chlamydia* have been described, of which the most prevalent is *Chlamydia pneumoniae*. Many studies have shown immunohistological and polymerase chain reaction (PCR) evidence of this infection in atherosclerotic coronary,<sup>1–4</sup> carotid,<sup>5–7</sup> and peripheral arteries.<sup>8</sup> In contrast, however, Weiss *et al* found no evidence of *C pneumoniae* by PCR or culture in 57 of 58 percutaneous coronary atherectomy specimens, or by electron microscopy in all of 22 specimens.<sup>9</sup> In a study from Australia, chlamydial DNA was undetectable by PCR in 49 atherosclerotic carotid and coronary artery samples from postmortems.<sup>10</sup> Moreover, given the demonstration by PCR of *C pneumoniae* DNA in

peripheral blood mononuclear cells of patients with coronary heart disease and healthy middle aged blood donors,<sup>11</sup> there is a lingering suspicion that this organism merely contaminates preformed atheromatous material.

More studies based on PCR are needed, but there are still a number of difficulties that must be overcome before these methods can be adopted widely for routine testing.<sup>12</sup> Current methods of human tissue sample preparation for amplification both of human and microbial DNA are problematic because Taq polymerase is readily inhibited by tissues. These problems are compounded by the unique life cycle of the chlamydiae,<sup>13</sup> the first stage of which, the elementary body, is highly condensed, owing to extensive disulphide crosslinking of cysteine rich envelope proteins, and must be lysed (for example, by incubation with detergents) to release chlamydial DNA. In itself, lysis of elementary bodies runs a risk of reducing DNA template activity.

There are several preparations available for PCR based analysis of *C pneumoniae* in solid tissue, but most are time consuming, labour intensive, or both. For example, whereas some investigators simply homogenise tissue and then treat the homogenate with detergent/proteinase K and heat,<sup>9</sup> others also perform phenol/chloroform extractions and ethanol precipitation,<sup>4,14</sup> or cetyltrimethylammonium bromide (CTAB) based purification of the nucleic acids before PCR.<sup>7</sup> Alternatively, samples may be homogenised directly in buffer containing detergent and RNase<sup>2,3,15</sup> and the resulting nucleic acids purified by conventional means<sup>15</sup> or by using commercial ion exchange based resins.<sup>2,3</sup>

By examining multiple preparation techniques, each designed to eliminate known classes of PCR inhibitors, the aim of our study was to compare methods of sample preparation to simplify and improve PCR based amplification from solid tissue.

## Methods

### TECHNIQUES FOR TISSUE PREPARATION

All arterial sections were stored at  $-20^{\circ}\text{C}$ . A tissue sample ( $\sim 3\text{ mm}^2$ ) containing both arterial wall and atheromatous plaque material was dissected from each section, which was returned immediately to  $-20^{\circ}\text{C}$ . The sample was then homogenised. Tissue dissections were performed under sterile conditions, using sterile, disposable implements. All sample preparations were performed in a laboratory separate to that in which the PCRs were performed. Filter tips for autopipettes were used throughout.

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Homogenisations were performed in sterile microcentrifuge tubes using sterile, disposable pestles (Sigma, Poole, Dorset, UK), either at room temperature in 80 µl of 1× Perkin Elmer PCR buffer (PE/Applied Biosystems, Warrington, UK) (PE buffer: 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin) or dry, over liquid nitrogen. Homogenised samples were then treated according to one or more of the following techniques.

GeneClean™ is a commercial preparation that purifies nucleic acid of proteins and other contaminants. In essence, a crude mixture containing DNA is added to fine glass beads under high salt conditions. Under these conditions, nucleic acids adhere to the glass beads, whereas contaminants such as proteins, carbohydrates, and ions do not. The beads are washed in a proprietary mixture of salts and ethanol to remove unbound, contaminating material. DNA is then eluted from the beads by a water wash because, under low salt conditions, nucleic acids do not adhere to the glass beads. GeneClean treatments were performed according to the manufacturer's instructions (Anachem, Luton, UK).

Proteinase K was dissolved in 1× PE buffer and added to homogenised samples to a final concentration of 10 µg/µl. Triton X-100 was added to a concentration of 0.5% and the mixture was incubated at 55°C for one hour. The enzyme was inactivated by heating to 95°C for five minutes. Samples were microfuged at 14 000 ×g for 30 seconds and the supernatant removed for use in PCR assays.

The Fe<sup>3+</sup> specific chelators ethylenediamine-N,N'-diacetic acid (EDDA) and deferoxamine mesylate (Sigma) were dissolved in 0.1 M NaOH to final concentrations of 10 mg/ml. Stock solutions were then added to the PCR mixtures, to final concentrations of 0.2 mg/ml, before cycling.

The lectin concanavalin A (Sigma) was dissolved in 1× PE buffer and added to the tissue homogenate to a concentration of 30 ng/µl. The mixture was incubated on ice for 30 minutes, with vortexing at five minute intervals. Samples were microfuged at 14000 ×g for 30 seconds and the supernatant removed for use in PCR assays.

For ultrafiltration, 15 µl of treated homogenate was processed through a Microcon®-30 microconcentrator (Millipore, Watford, UK) and the DNA was recovered from the upper reservoir according to manufacturer's instructions, in 10 µl sterile distilled H<sub>2</sub>O.

#### PCR AMPLIFICATIONS

PCRs (50 µl) were performed in either 1× PE buffer or 1× AB buffer IV (Advanced Biotechnologies, Epsom, UK) (AB buffer IV: 75 mM Tris/HCl, pH 9.0, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% Tween), with additional components as described, and either 1 µl of sterile distilled H<sub>2</sub>O (negative controls) or 1 µl of template DNA, prepared as described. Reactions were cycled 30 times for 30 seconds at 95°C, 30 seconds at 55°C, and 60 seconds at 72°C, unless stated otherwise. After amplification, 20% of each

reaction was examined by electrophoresis on a 2% agarose gel.

#### OPTIMISATION OF PCR AMPLIFICATIONS

PCR amplifications were optimised according to the following strategy:  
Buffer selection → Mg<sup>2+</sup> concentration → Quantity of Taq DNA polymerase → dNTP concentration → Primer concentration

#### MATERIALS

Genomic DNA was purified from *C pneumoniae* elementary bodies by Dr A Cunningham (University of Southampton, UK) and from *C psittaci* by Dr C Storey (University of Manchester, UK). Taq DNA polymerase was obtained from Perkin Elmer (PE/Applied Biosystems).

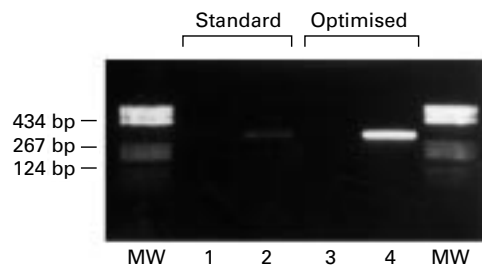
Oligonucleotide primers: CP1, 5'-GTGATC TGTAACGTATCG-3'; CP2, 5'-AGTAGA CAATGCTGTGGC-3'; H1, 5'-ACTCCT CGTTGTTTACCC-3'; and H2, 5'-TTC TCAGGATCCACATGC-3' were synthesised on a Beckman Oligo1000 DNA synthesiser or purchased from Applied Biosystems. Primers CP1 and CP2 anneal to the major outer membrane protein (MOMP) gene of *C pneumoniae* strain IOL207, as sequenced by Carter and colleagues<sup>16</sup> (Genbank accession number M64064). Primers CP1 and CP2 correspond to bases 670–687 and the reverse complement of bases 970–987 of sequence M64064, respectively. Primers H1 and H2 anneal to exon 2 of the human ε-globin gene, which is contained within the human β-globin region of chromosome 11 (Genbank accession number U01317). Primer H1 corresponds to the first 18 bases (19755–19772, U01317) and primer H2 corresponds to the reverse complement of bases 198–215 (19953–19970, U01317) of this exon.

Atheromatous plaque material was collected between September 1995 and September 1996. Patients were recruited after selection for carotid, coronary, or peripheral endarterectomy (for established atheromatous disease) by consultant vascular or cardiothoracic surgeons at the City Hospital, Birmingham and Walsgrave Hospital, Coventry. Exclusion criteria were known or suspected immunodeficiency or hepatitis B viral infection, or apparent risk of acquiring serum hepatitis or human immunodeficiency virus infection. All patients gave informed consent and the study was approved by the district ethics committee.

## Results

#### DEVELOPMENT OF PCR ASSAYS

Two PCR assays were developed to test the methods of sample preparation. One amplified hypervariable region IV of the *C pneumoniae* MOMP gene and the second was a simple amplification of human DNA, encompassing exon 2 of the human ε-globin gene. The first PCR is technically more demanding because of the need to lyse *C pneumoniae* elementary bodies. Conditions were therefore optimised for this amplification using the strategy outlined above, with purified *C pneumoniae* genomic DNA as a template.



**Figure 1** Optimisation of *Chlamydia pneumoniae* PCR. PCR amplifications of *H. O* (negative controls) and *C pneumoniae* genomic DNA were performed with primers CP1 and CP2 as described under standard (1× PE buffer, 100 μM dNTPs, 0.4 μM primers, 2 U Taq DNA polymerase) or optimised (1× AB buffer IV, 100 μM dNTPs, 0.6 μM primers, 4 U Taq DNA polymerase) conditions, as indicated. Lane 1, negative control; lane 2, *C pneumoniae* amplification; lane 3, negative control; lane 4, *C pneumoniae* amplification. MW, molecular weight marker (1 μg of a pBR322/HaeIII digest (Sigma)).

Amplification in a standard potassium containing buffer (Perkin Elmer) was compared with that in an ammonium sulphate containing buffer (Advanced Biotechnologies). Each buffer system was used with MgCl<sub>2</sub> at concentrations of 1.5 mM, 2.0 mM, 2.5 mM, and 3.0 mM. No appreciable difference was noted between the different magnesium concentrations, but amplification in the ammonium sulphate containing buffer gave a consistently greater yield of PCR product. The ammonium sulphate containing buffer with 1.5 mM MgCl<sub>2</sub> was therefore used in subsequent analyses.

Using this buffer system, the quantity of Taq DNA polymerase was optimised. PCR reactions with 1, 2, 3, 4, and 5 U of enzyme/reaction were performed. A great increase in product yield between 1 and 2 U and a minimal further increase between 2 and 4 U was observed. Therefore, 4 U of Taq DNA polymerase were used during the remaining optimisation reactions.

The nucleotide concentration was then optimised: dNTP concentrations of 20, 50, 100, 150, and 200 μM were tested. A large increase in PCR product yield was observed between 20 and 100 μM dNTPs, but no further increase in yield was observed above this point. A concentration of 100 μM dNTPs was therefore used for future assays.

Primer concentrations between 0.2 μM and 2 μM in 0.2 μM stages were then examined. Amplicon yield plateaued at a concentration of 0.6 μM of primers. Consequently, this concentration was used. Finally, the optimum annealing temperature was investigated.

Under the optimised conditions, PCRs with annealing temperatures of 45°C, 50°C, and 55°C were examined. Increasing temperature gave no diminution of product yield. The highest temperature (55°C) was therefore used in subsequent analyses. Figure 1 shows that a significant increase in amplicon yield was achieved.

Because there is appreciable homology between the MOMP genes of *C pneumoniae* and *C psittaci*,<sup>17</sup> a PCR under the optimised conditions was performed using purified *C psittaci* DNA as a template. No amplification was observed (data not shown).

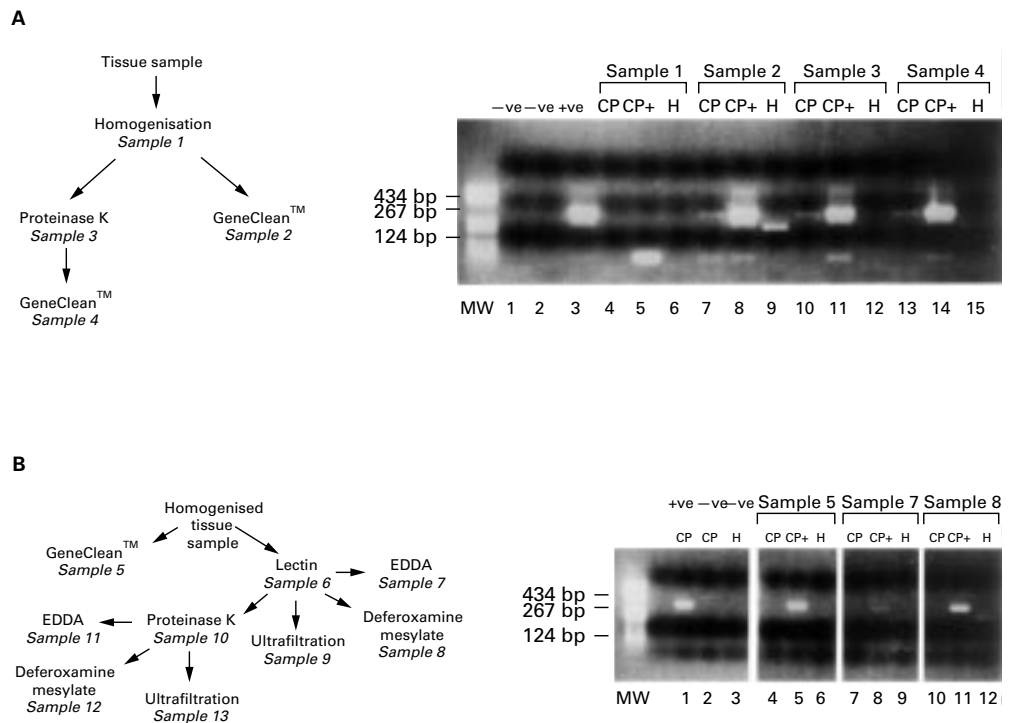
#### TECHNIQUES FOR TISSUE PREPARATION

Tissue preparation was evaluated initially using a single source of tissue. A section of atherosclerotic carotid artery was obtained by surgical endarterectomy in a patient with known carotid stenosis. Several preparative techniques were examined, to include one or more of the following: homogenisation, proteinase K treatment, GeneClean treatment, ultrafiltration, and the effects of lectin and Fe<sup>3+</sup> specific chelators.

Initially, a tissue sample was homogenised at room temperature in 1× PE buffer (sample 1). A 10 μl aliquot of this homogenate was then purified by GeneClean (sample 2). As a comparison, a 10 μl aliquot of the homogenate was treated with proteinase K (sample 3). Finally, a 5 μl aliquot of sample 3 was treated with GeneClean (sample 4). Each sample was then used as a template in three PCRs: amplification of the human ε-globin gene; amplification of the *C pneumoniae* MOMP gene; and a repeat of the *C pneumoniae* amplification, "spiked" with purified genomic *C pneumoniae* DNA (fig 2A). This last amplification served as a control, to examine the effectiveness of the preparative method in eliminating PCR inhibitors in the event that the sample contained no *C pneumoniae* DNA.

As illustrated in fig 2A, no specific DNA product (317 bp for the *C pneumoniae* amplicon and 215 bp for the human amplicon) was amplified from the untreated homogenate in any reaction. In contrast, positive, specific amplifications were seen from samples 2, 3, and 4 in all PCRs. However, the yield of PCR product was greatest from the template prepared by homogenisation and GeneClean (sample 2). Therefore, this preparation technique was used in subsequent assays. In addition to the specific amplicon, small DNA fragments (< 100 bp) were amplified in reactions that had been spiked with *C pneumoniae* genomic DNA and from the optimal sample preparation of the tissue sample (sample 2). Because the yield of these small products is very low in the presence of the specific amplicon, we suggest that they arise from a secondary priming event on *C pneumoniae* DNA, which occurs when specific amplification is impaired.

The chosen technique of homogenisation followed by GeneClean treatment was compared with alternative strategies. A fresh sample of tissue was homogenised as before and a 5 μl aliquot of the homogenate was treated with GeneClean (sample 5). Further 5 μl aliquots were treated with lectin alone (sample 6); lectin + EDDA (sample 7); lectin + deferoxamine mesylate (sample 8); lectin + ultrafiltration (sample 9); lectin + proteinase K (sample 10); lectin + proteinase K + EDDA (sample 11); lectin + proteinase K + deferoxamine mesylate (sample 12); and lectin + proteinase K + ultrafiltration (sample 13). No PCR products were obtained from any of the amplifications of samples 6 and 9–13 (data not shown), indicating that the treatments used to generate these samples had failed to remove PCR inhibitors. However,



**Figure 2** Summary of tissue preparation methods and their effectiveness for PCR amplifications. Tissue samples were prepared by homogenisation and supplementary treatments as indicated. Aliquots (1  $\mu$ l) of the prepared tissue samples were subjected to PCRs of the human  $\epsilon$ -globin gene (H); the *Chlamydia pneumoniae* major outer membrane protein (MOMP) gene (CP); and the *C pneumoniae* MOMP gene, in the presence of *C pneumoniae* genomic DNA (CP+) as indicated. MW, molecular weight marker; -ve, control in which 1  $\mu$ l H<sub>2</sub>O replaced the prepared tissue sample; +ve, control in which 1  $\mu$ l *C pneumoniae* genomic DNA replaced the prepared tissue sample. (A) Preparation methods and PCR analyses of samples 1–4; (B) preparation methods of samples 5–13 and PCR analyses of samples 5, 7, and 8.

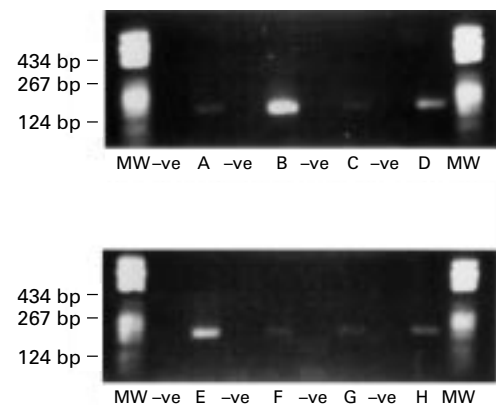
successful amplifications from samples 5, 7, and 8 were obtained (fig 2B). Although no native chlamydial DNA was detected in any of these samples, chlamydial DNA was successfully amplified in the spiked reactions, with a yield of: sample 5 > sample 8 > sample 7.

Collectively, these results demonstrate that either GeneClean or combined lectin/chelator treatment of the crude homogenate is sufficient to remove PCR inhibitors. Because the GeneClean treatment gave the highest yield of product in the spiked reaction and was shown to disrupt *C pneumoniae* elementary bodies (fig 2A), this method was used for future analyses. The lack of amplification of native chlamydial DNA in the second analysis (fig 2B) suggests either that this original tissue sample contained no *C pneumoniae*, or that the PCR reaction was insufficiently sensitive on this occasion.

#### PCR ANALYSIS OF CLINICAL SAMPLES

The sample preparation technique of tissue homogenisation followed by GeneClean treatment was applied to clinical samples from patients A–H, who were not known to be infected with *C pneumoniae*. Tissue samples were homogenised dry over liquid nitrogen and were then suspended in 80  $\mu$ l 1 $\times$  PE buffer and purified using a GeneClean Spin Kit™ (Anachem). Dry homogenisation and the GeneClean Spin Kit were introduced to minimise the potential for cross contamination between samples, whereas treatment of the entire homogenate was performed to improve the yield of template DNA. To monitor potential cross contamination, a blank homo-

genisation/purification containing only 1 $\times$  PE buffer was performed between that of each tissue sample. Each sample was then used as a template both for the *C pneumoniae* PCR and for PCR of the human  $\epsilon$ -globin gene. No *C pneumoniae* amplicon resulted from any of the samples (data not shown). However, successful amplification of human DNA was seen for all tissue samples (fig 3), confirming that tissue homogenisation followed by GeneClean treatment is an effective preparation of solid tissue for PCR.



**Figure 3** PCR amplification of human genomic DNA from tissue samples and blank preparations. Tissue samples A–H and interspersed blank aliquots of buffer were prepared by homogenisation and GeneClean™ treatment. PCRs of the human  $\epsilon$ -globin gene were performed on 1  $\mu$ l aliquots of each sample as indicated. -ve, interspersed blank preparations; MW, molecular weight marker.

## Discussion

*Chlamydia pneumoniae* is even more difficult to culture than the other chlamydiae, so epidemiological evidence relating to it derives mainly from serological studies.<sup>18</sup> In general, such studies have supported the view that *C pneumoniae* infection occurs throughout the world, in both affluent and deprived communities,<sup>19, 20</sup> in some of which it has been endemic at least since the late 1950s.<sup>21</sup> *Chlamydia pneumoniae* is a common respiratory pathogen in children as well as adults,<sup>22, 23</sup> and persistent asymptomatic infection also appears to be common.

Chronic coronary artery disease and acute myocardial infarction have been associated with serological evidence of *C pneumoniae* infection in many studies,<sup>24-30</sup> and other studies have demonstrated serological associations with cerebrovascular disease<sup>31, 32</sup> and systemic hypertension.<sup>33</sup> In particular, antibodies against the 40, 54, 60, 75, and 98 kDa outer membrane protein antigens of *C pneumoniae* appear to be associated strongly with atherosclerosis, implying that they may be virulence factors.<sup>34</sup> Serological evidence of previous *C pneumoniae* infection is also associated with a proatherogenic serum cholesterol profile.<sup>35</sup> *Chlamydia pneumoniae* has also been cultured from atherosclerotic material,<sup>36, 37</sup> and has been demonstrated by immunohistochemistry in three cell types known to be important in atherogenesis, namely: endothelial cells, macrophages, and smooth muscle cells,<sup>38</sup> all of which support *C pneumoniae* growth in vitro.<sup>39-41</sup> In animal models, chlamydiae disseminate at an early stage to peripheral blood mononuclear cells, spleen, and peritoneal macrophages,<sup>42, 43</sup> where they multiply.<sup>44</sup> Using PCR and immunocytochemistry, *C pneumoniae* has been shown to spread preferentially to cardiovascular tissue,<sup>45</sup> where infection is followed by arterial lesions that resemble atherosclerosis.<sup>46-48</sup> In cultured human endothelial cells, enhanced procoagulant (tissue factor) activity is also observed.<sup>49</sup>

This organism remains difficult to grow in the laboratory, and the usefulness of current culture and identification methods is limited by their sensitivity and specificity. Serology (principally micro-immunofluorescence) is still used widely, but crossreactions are reported between *C pneumoniae* and other chlamydiae, particularly *C psittaci*.<sup>50</sup> Crossreactions have also been reported with *Mycoplasma pneumoniae*, owing to contamination of a commercial *C pneumoniae* antigen preparation.<sup>51, 52</sup> Enzyme immunoassays have been developed for IgG and IgA antibodies to *C pneumoniae*, but there is no indication that their sensitivity and specificity are superior to those of micro-immunofluorescence.<sup>53</sup> Moreover, concentrations of antichlamydial IgG, IgA, and IgM antibodies measured in atherosclerotic lesions by micro-immunofluorescence may show no correlation with direct detection by PCR.<sup>54, 55</sup>

PCR of *C pneumoniae* can detect 10<sup>-16</sup> g of DNA, approximately equivalent to one chlamydial elementary body. It has been combined with enzyme immunoassay, and this

combined test (PCR-EIA) used to screen throat swab and bronchoalveolar lavage specimens for *C pneumoniae*.<sup>56, 57</sup> A two stage PCR has also been described, which detects *C psittaci* as well as *C pneumoniae* in the first stage, but amplifies only *C pneumoniae* DNA in the second stage.<sup>58</sup>

In our study, we have described two simple techniques for the preparation of solid tissue for PCR analysis. One, homogenisation followed by GeneClean treatment, has been performed on several tissue samples. The lack of contamination in blank samples suggests that this method is not prone to cross contamination. As an alternative, we have also described the preparation of tissue samples by homogenisation, followed by treatment with the Fe<sup>3+</sup> specific chelator deferoxamine mesylate.

In addition, we have developed an optimised PCR for the MOMP gene of *C pneumoniae* and demonstrated amplification of *C pneumoniae* DNA from a clinical sample. This result implies that our combined procedure of homogenisation and GeneClean treatment lyses *C pneumoniae* elementary bodies effectively. Interestingly, however, no *C pneumoniae* DNA was detected in a second sample of the same tissue. Although this result may be an artefact caused by insufficient sensitivity of the PCR reaction, it might also arise from patchy localisation of *C pneumoniae* within the arterial wall (analogous to localised bacterial colonies on the surface of an agar plate). Such a distribution of this organism would necessarily result in sporadic PCR results, because only some samples of the tissue would contain *C pneumoniae* DNA. Previous sporadic amplifications of *C pneumoniae* DNA from different sections of the same atherosclerotic lesion have been rationalised similarly.<sup>36</sup> No *C pneumoniae* DNA was amplified from clinical tissue samples A-H. This might have resulted either from the absence of *C pneumoniae* DNA in those samples or from insufficient sensitivity of the PCR reaction. However, in the context of previous PCR based studies,<sup>9, 10</sup> failure to detect *C pneumoniae* in only eight atheromatous plaques is unsurprising. The successful amplification of human DNA from all clinical samples demonstrates that tissue homogenisation followed by GeneClean treatment is an effective preparation of solid tissue for PCR.

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## Simplified preparation of human arterial sections for PCR analysis of *Chlamydia pneumoniae* and human DNA.

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