Multiple genotypes of *Chlamydia pneumoniae* identified in human carotid plaque

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*Chlamydia pneumoniae* is an obligate intracellular respiratory pathogen that causes 10 % of community-acquired pneumonia and has been associated with cardiovascular disease. Both whole-genome sequencing and specific gene typing suggest that there is relatively little genetic variation in human isolates of *C. pneumoniae*. To date, there has been little genomic analysis of strains from human cardiovascular sites. The genotypes of *C. pneumoniae* present in human atherosclerotic carotid plaque were analysed and several polymorphisms in the variable domain 4 (VD4) region of the outer-membrane protein-A (*ompA*) gene and the intergenic region between the *ygdD* and uridine kinase (*ygdD–urk*) genes were found. While one genotype was identified that was the same as one reported previously in humans (respiratory and cardiovascular), another genotype was found that was identical to a genotype from non-human sources (frog/koala).

While *C. pneumoniae* has been thought of primarily as a human-specific pathogen, several studies have now shown that *C. pneumoniae*-like stains are also present in a range of animals, including horses (Storey et al., 1993), koalas (Bodetti & Timms, 2000; Wardrop et al., 1999), reptiles and amphibians (Berger et al., 1999; Bodetti et al., 2002; Reed et al., 2000). Of the animal infections reported, a total of four non-human genotypes of *C. pneumoniae* have been described (Bodetti et al., 2002; Girjes et al., 1994; Glassick et al., 1996; Storey et al., 1993; Wardrop et al., 1999). These non-human isolates all appear to have relatively minor, but potentially important, sequence differences when compared to the published human genotypes (99.2–99.4 % homology). These sequence differences range from 1 bp variation over 342 bp for the *groESL* gene (human versus koala) to 21 bp variations over 279 bp for the *ompAVD4* gene (human versus horse) (Wardrop et al., 1999). A single report has found that various reptiles and amphibians are infected with the same or very similar genotype of *C. pneumoniae* as humans, as the samples were 100 % identical at the 16S

INTRODUCTION

*Chlamydia pneumoniae* is an obligate intracellular respiratory pathogen that causes 10 % of community-acquired pneumonia (Grayston et al., 1990) and is strongly associated with cardiovascular disease (Jackson et al., 1997; Maass et al., 1998a; Ramirez, 1996; Rassu et al., 2001; Shor et al., 1998). Saikku et al. (1988) first linked this bacterium to atherosclerosis, as *C. pneumoniae*-specific antibodies were detected during screening for probable infectious agents in carotid artery disease (CAD) patients. The results showed that people infected with *C. pneumoniae* were 2-6 times more likely to develop atherosclerosis than uninfected CAD patients. Since then, over 90 publications have reported the detection of *C. pneumoniae* in atherosclerotic arteries by various techniques, including electron microscopy (Shor et al., 1998), immunohistochemical staining (Cochrane et al., 2001), PCR (Maass et al., 1998b; Rassu et al., 2001) and culture (Jackson et al., 1997; Maass et al., 1998a; Ramirez, 1996). *C. pneumoniae* has been detected in approximately 60 % of atherosclerotic arteries compared to only 3 % of granulomatous and control arterial tissue, suggesting that the bacterium displays an important tropism to atherosclerotic arterial tissue (Taylor-Robinson & Thomas, 1998). More specifically, *C. pneumoniae* has been detected in all cells involved in atherosclerotic lesion formation, including macrophage-derived foam cells, smooth muscle cells (Shor & Phillips, 2000) and endothelial cells (Gaydos et al., 1996), as well as peripheral blood mononuclear cells (PBMCs) (Leowattana et al., 2001; Sessa et al., 2001) and T lymphocytes (Ezzahiri et al., 2002; Haranaga et al., 2001) that may potentially serve as vehicles for systemic dissemination. Research conducted on animal models has also supported the ‘infectious’ hypothesis for atherosclerosis, as repeated respiratory inoculations with *C. pneumoniae* have induced the formation of early atherosclerotic lesions and accelerated atherogenic progression in mice, rabbits and pigs (Blessing et al., 2001; Ezzahiri et al., 2002; Fong et al., 1999; Pislaru et al., 2003).

Abbreviations: CAD, carotid artery disease; PBMCs, peripheral blood mononuclear cells; VD4, variable domain 4.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are: CpnB, AY426606; CpnC, AY426607; CpnII, AY427827.
rRNA gene and ompA loci (Bodetti et al., 2002). In the present study, we used PCR and genotyping of the C. pneumoniae positives from patients with CAD to test the hypothesis that infected individuals have a single genotype of C. pneumoniae present in their plaque and that this genotype is common among other CAD patients.

METHODS

The study was conducted with the approval of the Queensland University of Technology, Human (1736H) and Animal (14/13/1A) Ethics Committees.

Human CAD samples. This study analysed a subset of 10 patients from a larger cohort of 54 CAD patients who were undergoing elective surgery (carotid endarterectomy). Atherosclerotic plaques removed at the time of surgery were immediately fixed in 10% formaldehyde and later decalcified and paraffin-embedded. DNA was extracted from 30 µm-thick sections of paraffin-embedded carotid tissue from each patient using the QIAamp Tissue Kit (Qiagen). The 60 µl of resultant DNA extract was stored at −70°C until tested. In six patients, multiple sections from the same carotid plaque were tested to confirm the presence of the bacteria throughout the atherosclerotic carotid artery.

Human PBMC specimens. From 43 individuals from the same cohort of CAD patients, the PBMC fraction was separated from 8 ml of venous blood using Vacutainer CPT cell preparation tubes (Becton Dickinson). The mononuclear cells were isolated, washed twice with PBS and resuspended in 0-5 ml PBS. The samples were heat-treated at 100°C for 10 min and stored at −70°C until tested.

Non-human C. pneumoniae genotypes. Given that our results showed that some of the human samples we analysed harboured a genotype of C. pneumoniae that is similar/identical to those previously found in koalas, we subsequently genotyped additional strains of C. pneumoniae from a previous koala study in our laboratory to examine whether only non-human strains of C. pneumoniae are present in koalas (Bodetti & Timms, 2000).

ompA PCR. The VD4 region of the ompA gene was selected as a target for genotyping, as the maximum variability between non-human and human isolates of C. pneumoniae has been reported in this small region (Wardrop et al., 1999). C. pneumoniae DNA within human carotid specimens and within PBMCs from humans and koalas was detected using a nested PCR assay targeting a 366 bp fragment of the C. pneumoniae ompA gene (Bodetti & Timms, 2000). The nested PCR used was highly specific, and could detect down to 10 chlamydial bodies. For both rounds, DNA (minimum concentration 10 ng per PCR) was amplified in 25 µl volumes containing 1 µl of primers, 200 µM deoxyribonucleotides, 1 × PCR buffer, 1-2 U Taq polymerase and 1 µl DNA sample or first-round template. Each sample was tested in duplicate and run in conjuction with controls: positive controls containing 100 copies of C. pneumoniae 446 bp first-round PCR product, and negative controls with no template added.

ygeD–urk PCR. The C. pneumoniae strains CWL029 and AR39 differ in the plasticity zone between the ygeD and urk genes by the orientation of a 23 bp sequence (Read et al., 2000). Therefore, a 568 bp fragment spanning this target region was amplified using a nested PCR. Only the human C. pneumoniae reference strains and the clinical samples that were 100% identical at the ompA locus to the koala type 1 strain were analysed. A 1 µl volume of each sample was added to a 25 µl reaction mix containing 1 µl of each primer (5’GTTAGGGTTGGTTTCCAGGC3’ and 5’GAGATAACGATTCTGAGGCC3’), 1 × Boehringer Mannheim PCR buffer, 200 µM each deoxyribonucleotide (Boehringer Mannheim) and 1-2 U Taq polymerase (Boehringer Mannheim). Cycling conditions consisted of an initial denaturation for 5 min at 95°C, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension for 1 min at 72°C, and a final extension for 10 min at 72°C. For second-round PCR, 1 µl of the first-round product was mixed with 25 µl of the above amplification mixture, using the nested primers 5’CCAGAACCTCGTAATTC3’ and 5’GCTGTTTCTACAGGAAAATGACC3’. The PCR parameters were kept constant, except for the annealing temperature of 50°C. Each sample was tested in conjunction with controls: negative controls with no template added, and positive controls containing 100 copies of C. pneumoniae first-round PCR product. Negative controls were included after every third test sample to check for aerosol contamination. The PCR products were visualized by ethidium bromide staining following electrophoretic separation.

Genotyping. ompA and ygeD–urk PCR products were separated by gel electrophoresis and excised from the gel using a commercial Gel Extraction Kit (Qiagen). Sense and anti-sense strands were sequenced using BigDye Terminator Chemistry in an ABI 3730xl automatic capillary DNA sequencer (Australian Genome Research Faculty, Brisbane, Australia). All samples were confirmed as C. pneumoniae using a BLAST search at http://www.ncbi.nlm.nih.gov/blast. ‘eclustalw’ in WebANGIS was used to align the sequences of the test samples and to compare the results to various reference C. pneumoniae isolates, including AR39, CM1, CV3, CWL029, IOL207, J138, TW10, TW183, VR1356 and WA97001. AR39, WA97001 and Koala type 1 sequences were obtained from the GenBank database, while all other isolates were genotyped in our laboratory from culture-propagated material.

Cloning. Any carotid plaque specimen that harboured more than one genotype of C. pneumoniae was cloned to determine the ratio of each genotype present. The pGEM-T Easy Vector System was employed for ligation and transformation of the PCR products into JM109 competent cells, as per the manufacturer’s protocol (Promega). For each PCR reaction, 10 individual clones were genotyped.

RESULTS AND DISCUSSION

Human CAD specimens: ompA VD4 genotyping

The VD4 region within the ompA gene of C. pneumoniae was genotyped in 10 human carotid atherosclerotic artery plaque specimens and compared to the gene sequences of 10 strains of C. pneumoniae (Table 1). J138 was chosen as the reference sequence for comparison (designated genotype A). The human isolates J138, AR39, CM1, CWL029, IOL207, TW10, TW183, VR1356 and WA97001 were 100% identical at this locus, and are all referred to as genotype A (Table 1). The 342 bp segments of the ompAs that were sequenced from huCAD1, huCAD3, huCAD5 and huCAD7 were 100% identical to each other and to genotype A. Three carotid samples (huCAD2, huCAD4 and huCAD10) had single silent polymorphisms, at position 18029 (T to C) for huCAD10 (designated genotype B; accession number AY426606) and position 180311 (A to G) for huCAD2 and huCAD4 (designated genotype C; accession number AY426607; Fig. 1).

The remaining three human carotid samples (huCAD6, huCAD8 and huCAD9) all varied from J138 by 6 bp and
were designated ompA genotype D (Fig. 1). The duplicate sample of huCAD1 was infected with *C. pneumoniae* genotype D. This genotype D sequence was the same as that previously reported from koalas (Wardrop et al., 1999). The six nucleotide differences between genotype D and the other genotypes (A, B and C) in the VD4 region of the *ompA* gene (342 bp), translate into four amino acid changes.

The detection of *C. pneumoniae* in human carotid atheromas supports a role for this organism in atherogenesis. Previous studies have shown that the *C. pneumoniae* strains infecting humans appear to be almost clonal, as the available sequence data show nearly identical sequences for *ompA, ompB*, the 16S rRNA gene, domain I of the 23S rRNA gene, the 16S–23S rDNA intergenic spacer, Rnase P RNA, *groEL*, *dnaK* and *waaA* (*kdtA*) (Everett & Andersen, 1997; Gaydos et al., 1992a,b; Herrmann et al., 1996; Jackson et al., 1997; Jantos et al., 1997; Kuo et al., 1993; Maass et al., 1998a; Molestina et al., 1998; Pettersson et al., 1997; Read et al., 2000; Shirai et al., 2000a,b). Only three studies have genotyped samples obtained from plaque material (Kuo

### Table 1. Genotypes of *C. pneumoniae* detected in human and koala clinical samples

<table>
<thead>
<tr>
<th>Samples</th>
<th><em>C. pneumoniae</em> genotype: designed by differences at the <em>ompA</em> VD4 locus (342 bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (0 bp)</td>
</tr>
<tr>
<td>Reference strains (<em>n</em>=10)</td>
<td>AR39*, CM1, CWL029, IOL207, J138*, TW10, TW183, VR1356, WA97001</td>
</tr>
<tr>
<td>Human clinical samples (CAD, <em>n</em>=10; PBMC, <em>n</em>=1)</td>
<td>huCAD1†, 3, 5, 7</td>
</tr>
<tr>
<td>Koala clinical samples (<em>n</em>=4)</td>
<td>KoPBMC2, 3, 4</td>
</tr>
</tbody>
</table>

*Sequence obtained from GenBank.
†Multiple genotypes displayed in one patient.

Fig. 1. Nucleotide sequence alignment of a 342 bp fragment of the *ompA* gene (VD4 region) for 27 *C. pneumoniae* isolates. Dashes indicate residues identical to those of the *ompA* loci of the *C. pneumoniae* J138 strain represented as CpnA. CpnA = J138, AR39, CM1, CWL029, CWL050, IOL207, TW10, TW183, VR1356, huCAD1, huCAD3, huCAD5, huCAD7, KoPBMC2, KoPBMC3 and KoPBMC4; CpnB = huCAD10; CpnC = huCAD2 and huCAD4; CpnD = Koala type 1, huCAD1, huCAD6, huCAD8, huCAD9, huPBMC1 and KoPBMC1; CpsK = *C. psittaci* isolated from a koala (Girjes et al., 1994). GenBank accession numbers: CpnB, AY426606; CpnC, AY426607.
et al., 1993; Maass et al., 1998a; Ramirez, 1996). Our study was the first to genotype a significant number of samples from plaque, and unexpectedly showed that polymorphisms do exist between cardiovascular \textit{C. pneumoniae} sources. Even though a limited amount of sequence was analysed, three of the genotypes observed were identical to previously identified human strains, while one was of animal origin, being the same genotype as that previously identified in both a frog and koalas (Berger et al., 1999; Bodetti & Timms, 2000; Wardrop et al., 1999). Interestingly, \textit{ompA} genotypes B, C and D appear to be geographically restricted to Australia, unlike genotype A, which has a worldwide distribution.

**Human and koala PBMC specimens: \textit{ompA} genotyping**

Additional genotyping was performed on human PBMCs to determine whether genotypes A–D of \textit{C. pneumoniae} are restricted to vascular sites: \textit{C. pneumoniae} was detected in two of the 43 human PBMC fractions using the \textit{ompA} nested PCR procedure. One of these samples (huPBMC1) was able to be genotyped and was found to be \textit{ompA} genotype D.

Given that human samples harboured a genotype of \textit{C. pneumoniae} that is similar/identical to those previously found in koalas, this study examined whether only non-human strains of \textit{C. pneumoniae} are present in koalas. Of the four koala-PBMC specimens analysed, one was infected with \textit{ompA} genotype D and the other three were infected with \textit{ompA} genotype A (Table 1). Therefore, genotypes D and A of \textit{C. pneumoniae} are not restricted solely to humans or koalas.

The chlamydial strains of koala origin have previously shown minor sequence differences to the strains from human origin at several loci, including the \textit{groESL} intergenic region, the16S rRNA gene, \textit{ompB} and the VD4 region of the \textit{ompA} gene (Glassick et al., 1996; Wardrop et al., 1999). This is the first report of koala PBMCs being infected with a genotype of \textit{C. pneumoniae} that is also found in humans. Other animals have previously been reported to harbour chlamydial infections with a genotype similar to A, including frog (Australia), turtle (USA), python (USA and Europe), puff adder (USA) and iguana (Central America) (Bodetti et al., 2002).

**Multiple genotypes present in a single patient: \textit{ompA} genotyping**

Multiple sections of atherosclerotic carotid material (\( n = 26 \)) from six CAD patients were tested separately, and each section was genotyped (Table 2). In the case of five patients (huCAD5, huCAD6, huCAD7, huCAD8 and huCAD9), the same genotype [huCAD5, huCAD7 (= genotype A), huCAD7, huCAD8, huCAD9 (= genotype D)] was detected in all of the subsamples analysed (two to seven sections per patient). A dual infection with two different genotypes of \textit{C. pneumoniae} (genotypes A and D) was present within atherosclerotic plaque from one human CAD patient (huCAD1). Interestingly, the two different genotypes were not found in the same 30 µm section of the artery. To study the distribution of the \textit{C. pneumoniae} genotypes in huCAD1, we produced 10 clones for each of two 30 µm sections of the carotid plaque. All 10 clones produced the same genotype (A or D).

The two \textit{C. pneumoniae} genotypes within the same carotid plaque (genotypes A and D) may have infected the patient (huCAD1) on different occasions, as reinfection with \textit{C. pneumoniae} is common. Other studies have shown that cell-mediated and humoral immune responses towards \textit{Chlamydia} are only partial and are usually short-lived (Ward, 1995). In addition, continual reexposure to the organism appears to lead to a hypersensitivity response that is associated with inflammation and tissue injury, both of which are characteristics of atherosclerosis.

**\textit{ygeD–urk} genotyping**

The 23 bp invertible region in the \textit{C. pneumoniae} plasticity zone is in the forward orientation in eight reference strains (J138, CM1, CWL029, IOL207, TW10, TW183, VR1356 and WA97001; designated genotype I) and in the reverse orientation in one of the strains, AR39 (designated genotype II). Apart from this inversion, no other polymorphisms were identified within the 371 bp PCR product of the 10 human reference strains. The four human carotid (huCAD1, huCAD6, huCAD8, huCAD9) and one koala PBMC (KO) specimens that were found to harbour \textit{C. pneumoniae} of genotype D all had the 23 bp inverted region in the same orientation as J138 and had 3 bp differences within the \textit{ygeD–urk} region at positions 224938 (C to T), 225044 (C to T) and 225072 (T to C; designated genotype III; accession number AY227827; Fig. 2).

Interestingly two \textit{C. pneumoniae} genotype A and D/III strains are not solely restricted to humans or koalas. This finding suggests that these strains are potentially not host

**Table 2. Presence of multiple genotypes of \textit{C. pneumoniae} in human CAD plaque samples**

Genotypes A and D were designated on the basis of sequence differences. J138 was chosen for sequence comparisons and designated genotype A. Genotype D was previously reported from an ocular isolate from koalas.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sections tested</th>
<th>\textit{C. pneumoniae} \textit{ompA} genotype</th>
</tr>
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<tbody>
<tr>
<td>huCAD1</td>
<td>3</td>
<td>A: 1, D: 2</td>
</tr>
<tr>
<td>huCAD5</td>
<td>4</td>
<td>A: 4, D: 0</td>
</tr>
<tr>
<td>huCAD6</td>
<td>7</td>
<td>A: 0, D: 7</td>
</tr>
<tr>
<td>huCAD7</td>
<td>2</td>
<td>A: 2, D: 0</td>
</tr>
<tr>
<td>huCAD8</td>
<td>4</td>
<td>A: 0, D: 4</td>
</tr>
<tr>
<td>huCAD9</td>
<td>6</td>
<td>A: 0, D: 6</td>
</tr>
</tbody>
</table>

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specific. The possibility of amplicon contamination has been considered, but contamination was highly unlikely in our study as: two different target genes (ompA and ygeD–urk) were analysed; multiple sections were analysed for reproducibility; DNA extraction, PCR preparation, PCR amplification and product analyses were conducted in physically separate areas; all equipment was isolated to each area so that the DNA-free environment for PCR preparation was maintained; fresh filter-tips were used when manipulating all samples to prevent possible contamination via aerosols; and DNA negative controls remained negative for each PCR run. Each sample was amplified twice and sequenced twice, thereby ensuring that the minor polymorphisms we observed were not PCR artefacts.

In summary, specific gene typing of the C. pneumoniae ompA and ygeD–urk genes showed that there are minor polymorphisms present in C. pneumoniae strains infecting human atherosclerotic carotid plaque. Five different genotypes of C. pneumoniae were reported (genotypes A/I, A/II, B, C and D). Interestingly, two of these genotypes were of non-human origin, having now been reported in both humans and koalas. Our findings suggest that C. pneumoniae may be capable of being transmitted between humans and animals, although the origin and direction of transmission is presently unknown.

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