Molecular characterization of *Chlamydophila pneumoniae* isolates from Western barred bandicoots

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Chlamydophila pneumoniae is an obligate intracellular respiratory pathogen that has been associated with pneumonia and chronic bronchitis, atherosclerosis, asthma and other chronic diseases in humans. However, *C. pneumoniae* is not restricted to humans, as originally thought, and can cause infections in several animal hosts. *C. pneumoniae* was isolated in cell culture from nine Western barred bandicoots (*Perameles bougainville*) from Australia. The sequences of five genomic regions were determined, including full-length sequences of the16S rRNA and *ompA* genes and the *ygeD–urk* intergenic spacer, and partial sequences of the 23S rRNA and *rpoB* genes. Sequence analysis of the entire 16S rRNA and *ompA* genes from bandicoot isolates demonstrated that they were 98.2–98.3 % similar to human isolates, 94.6–99.3 % similar to the equine biovar and almost identical, with 99.5–99.9 % similarity, to the koala biovar. Comparative genotyping of the variable domain 4 region of the *ompA* gene demonstrated that bandicoot isolates seemed to be identical to the animal genotype that has been recently identified in human carotid plaque specimens. Minor sequence polymorphism observed in *ompA*, 16S rRNA and *rpoB* genes of animal isolates, indicating genomic diversity within *C. pneumoniae*, may have important implications for diagnostic PCR assays leading to false negative results. Forty percent of selected published species-specific PCR assays were found to have sequence variability in primer and/or probe that might affect their performance in detecting bandicoot isolates of *C. pneumoniae*, or possibly other animal and human strains where minor sequence polymorphisms may be present. The data from this study support the previous observations that *C. pneumoniae* is not restricted to humans and may be widespread in an animal reservoir with a potential risk of transmission to humans.

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INTRODUCTION

Chlamydophila pneumoniae is an obligate intracellular bacterium responsible for respiratory infections (pneumonia and bronchitis) in adults and children, affecting up to 70 % of the worldwide population at least once during his or her lifetime (Peeling & Brunham, 1996). Persistent *C. pneumoniae* infections have been implicated in the development of atherosclerosis, asthma and other chronic diseases in humans (Balin *et al.*, 1998; Blasi *et al.*, 2002; Hahn *et al.*, 1991; Wong *et al.*, 1999).

C. pneumoniae was initially thought to be an exclusively human pathogen. However, several studies demonstrated that *C. pneumoniae* could also cause ocular, respiratory and urogenital infections in a wide variety of animal species, including koalas, horses, frogs and reptiles (Berger *et al.*, 1999; Bodetti *et al.*, 2002; Hotzel *et al.*, 2001; Jacobson *et al.*, 2004; Storey *et al.*, 1993). Current taxonomic classification divides *C. pneumoniae* into three distinct biovars: human biovar TWAR, koala biovar and equine biovar (Everett *et al.*, 1999a). The distinction was based primarily on comparative sequence analysis of the 16S rRNA, 23S rRNA and *ompA* genes, and biological characteristics. Human *C. pneumoniae* isolates were found to be almost identical to each other, with only 0.1 % difference in 16S rRNA gene and 0.4 % difference in the *ompA* gene. While some of the animal isolates are

Abbreviations: SNP, single nucleotide polymorphism; WBB, Western barred bandicoot.

The GenBank/EMBL/DDBJ accession numbers for the *ompA* and 16S rRNA genes, partial sequences of 23S rRNA and *rpoB* genes, and *ygeD–urk* intergenic spacer from bandicoot *C. pneumoniae* isolates are DQ358972, DQ444323, DQ465990, DQ460031 and DQ463439, respectively.

almost identical to human strains, the others seem to be genetically more diverse, with up to 6 % *ompA* gene dissimilarity (Bodetti *et al.*, 2002).

The existence of animal strains of *C. pneumoniae* raises the issue of possible transmission risk to humans. A recent study by Cochrane *et al.* (2005), where animal genotypes of *C. pneumoniae* were identified in human specimens, and human genotypes were detected in koalas, supports the possibility of such transmission. However, no cases of zoonotic *C. pneumoniae* infections in humans have been described so far.

In this study, *C. pneumoniae* was isolated from Western barred bandicoots (WBBs) (*Perameles bougainville*) in cell culture and characterized by sequence comparison of five genomic regions relevant for molecular diagnostics, including 16S rRNA, 23S rRNA, *ompA* and *rpoB* genes and the *ygeD–urk* intergenic spacer. The entire *ompA* and 16S rRNA genes, which are frequently used in species-specific PCR-based assays (Dowell *et al.*, 2001; Loens *et al.*, 2006), were sequenced and compared to the published sequences of human and animal *C. pneumoniae* isolates. Genotyping of the bandicoot isolates was performed in the variable domain 4 of the *ompA* gene, *rpoB* gene and *ygeD–urk* intergenic spacer loci.

METHODS

Animals. All WBBs sampled in this study were from wild populations from Bernier Island and Dryandra, Western Australia. Thirty-seven conjunctival, nasal, throat and cloacal swab specimens from twenty-one animals with clinical signs of ocular and/or respiratory disease were examined.

Isolation and propagation of *Chlamydiales*. Isolation and propagation of *Chlamydiales* was performed by cell culture, as previously described (Roblin *et al.*, 1992). Briefly, swab specimens were inoculated onto HEp-2 cell monolayers (ATCC CCL-23) by centrifugation at 1700 ***g*** for 1 h. Infected monolayers were then overlaid with Iscove's DMEM medium (Sigma-Aldrich) supplemented with 10 % fetal calf serum (Atlanta Biologicals), 20 mM L-glutamine and 1 μg cycloheximide ml^{-1}, and incubated at 35 °C for 72 h. Up to six passages were performed for each isolate. Following incubation, inoculated HEp-2 cells were fixed in ethanol and stained for specific chlamydial inclusions with the Pathfinder *Chlamydia* culture confirmation system (Bio-Rad) for 30 min at 37 °C.

DNA extraction. DNA from the cultured *Chlamydiales* was extracted using a DNeasy Tissue kit (Qiagen) according to the manufacturer's protocol. Human *C. pneumoniae* CWL029 (ATCC VR-1310) and TW-183 (ATCC VR-2282) grown in HEp-2 cells were used as a positive control in PCR assays.

***Chlamydiaceae*-specific PCR TaqMan assay.** The TQF and TQR primers and probe targeting the *Chlamydiaceae*-specific region of the 23S rRNA gene were as described by Everett *et al.* (1999b) (Table 1). PCR was performed using a LightCycler 2.0 (Roche) system with DNA Master HybProbe kit (Roche), containing FastStart *Taq* DNA polymerase, under the following conditions: an initial denaturation step at 95 °C for 10 min, then 45 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 10 s and extension at 72 °C for 10 s. Product amplification was analysed with the manufacturers' supplied software.

***C. pneumoniae*-specific PCR TaqMan assay.** The primers QMOMP1, QMOMP2 and QMOMPS probe targeting an 85 bp *C. pneumoniae*-specific region of the *ompA* gene are shown in Table 1. PCR was performed using the Roche system mentioned above, with predenaturation at 95 °C for 10 min, then 45 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 10 s and extension at 72 °C for 10 s.

16S and 23S rRNA genes signature sequencing. Amplification/sequencing primers used for 16S rRNA gene and 23S rRNA gene products were 16SIGF, 16SIGR, U23F and 23SIGR (Table 1). PCR was performed using Qiagen ProofStart DNA polymerase kit (Qiagen), with initial denaturation at 95 °C for 5 min, then 45 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 60 s, with a final extension for 5 min at 72 °C. Following amplification, the products were separated by electrophoresis in 2 % agarose E-gel (Invitrogen) and visualized using an ultraviolet transilluminator. PCR products were purified with a QIAquick PCR purification kit (Qiagen) and sequenced in both directions (GeneWiz).

***ompA* gene sequencing.** A DNA fragment containing the entire *ompA* gene was amplified with CpompA1F and CpompA3R primers (Table 1). PCR was performed using a ProofStart DNA polymerase kit, with an initial denaturation step at 95 °C for 5 min, then 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 60 s, with a final extension for 5 min at 72 °C. Following electrophoresis in 2 % agarose E-gel, PCR products were visualized by using an ultraviolet transilluminator. PCR products containing 1170 bp *ompA* gene were sequenced in both directions, with overlap, using the sequencing primers shown in Table 1. These primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Rozen & Skaletsky, 2000).

16S rRNA gene sequencing. The DNA region containing the entire 16S rRNA gene was amplified with Cp16SF and Cp16SRb primers (Table 1). PCR was performed using a ProofStart DNA polymerase kit, with initial denaturation step at 95 °C for 5 min, then 45 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 60 s, with a final extension for 5 min at 72 °C. Following electrophoresis in 2 % agarose E-gel, PCR products were visualized and purified with QIAquick PCR purification kit. PCR products containing the 1552 bp 16S rRNA gene were sequenced in both directions, with overlap, using the following primers: Cp16SF1, Cp16SR1, Cp16SF2, Cp16SR2, Cp16SF3, Cp16SR3, Cp16SF4 and Cp16SR4 (Table 1). The above primers were designed using the Primer3 program.

***rpoB* gene sequencing.** A 733 bp section of the DNA-directed RNA polymerase *β* gene (*rpoB*) containing segment, matching a *C. pneumoniae*-specific *Pst*I fragment (Campbell *et al.*, 1992), was amplified using CprpoBFc and CprpoBRc primers (Table 1), designed using Primer3. PCR was performed using a ProofStart DNA polymerase kit, with the initial step at 95 °C for 5 min, then 45 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 60 s, with a final extension for 5 min at 72 °C. PCR products were purified and sequenced in both directions using the same primer pair.

***ygeD–urk* intergenic spacer sequencing.** A 621 bp fragment spanning the *ygeD–urk* intergenic spacer was amplified and sequenced using OUT1 and OUT2 primers (Table 1). PCR was performed using a ProofStart DNA polymerase kit under the above conditions. Prior to sequencing products were purified from 2 % agarose E-gels with a QIAquick PCR purification kit.

Sequence analysis. The sequences were analysed using BLAST 2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/) (Tatusova & Madden,

Table 1. Oligonucleotides used in this study

Name	Target gene	Sequence	Primer location (*C. pneumoniae* TW-183 genome numbering)	Application	Product size (bp)	Reference
QMOMP1	*ompA*	5′-GATCCGCTGCTGCAAACTATACT-3′	176626–176648	Real-time PCR	85	Apfalter *et al.* (2003)
QMOMP2		5′-GTGAACCACTCTGCATCGTGTAA-3′	176688–176710	Real-time PCR		
probe		5′-FAM-TAGGCCGGGTTAGGTCTATCTACGGC-AGT-TAMRA-3′	176649–176677	Real-time PCR		
CpompA1F	*ompA*	5′-TCGTGTCGCCAAAATATGAG-3′	176283–176302	PCR/sequencing	1344	This study
CpompA3R		5′-GGGGGAATAAAAATTGCTCA-3′	177607–177626	PCR/sequencing		
CpompA1R	*ompA*	5′-CCAACGAGATTGAACGCTGT-3′	176802–176821	Sequencing	–	This study
CpompA2F		5′-GCAGGCTTCATTGCCTTAAA-3′	176715–176734	Sequencing		
CpompA2R		5′-GCGGATGTTATCAGCATCAA-3′	177235–177254	Sequencing		
CpompA3F		5′-GGAACAAAGTCTGCGACCAT-3′	177123–177142	Sequencing		
16SIGF	16S rRNA	5′-CGGCGTGGATGAGGCAT-3′	96733–96749	PCR/sequencing	294	Everett *et al.* (1999a)
16SIGR		5′-TCAGTCCCAGTGTTGGC-3′	97010–97026	PCR/sequencing		
Cp16SF	16S rRNA	5′-GATGTGGATACGCAACGAAA-3′	96550–96569	PCR	1796	This study
Cp16SRb		5′-TGAAAACAACCTTAACAATGCAA-3′	98323–98345	PCR		
Cp16SF1	16S rRNA	5′-TGCAGGCCAGTATAAAATGC-3′	96634–96653	Sequencing	–	This study
Cp16SR1		5′-TAAGGCCTTCATCACACACG-3′	97104–97123	Sequencing		
Cp16SF2		5′-ACACTGCCCAGACTCCTACG-3′	97028–97047	Sequencing		
Cp16SR2		5′-CATCGTTTACGGCAAGGACT-3′	97503–97522	Sequencing		
Cp16SF3		5′-GAAAGCAAGGGGAGCAAAC-3′	97466–97484	Sequencing		
Cp16SR3		5′-CCATTGTAGCACGTGTGTCG-3′	97924–97943	Sequencing		
Cp16SF4		5′-ATGACGTCAAGTCAGCATGG-3′	97889–97908	Sequencing		
Cp16SR4		5′-CCCAACCTAGTCGGGTTGTT-3′	98272–98291	Sequencing		
TQF	23S rRNA	5′-GAAAAGAACCCTTGTTAAGGGAG-3′	98986–99008	Real-time PCR	128	Everett *et al.* (1999b)
TQR		5′-CTTAACTCCCTGGCTCATCATG-3′	99092–99113	Real-time PCR		
probe		5′-FAM-CAAAAGGCACGCCGTCAAC-TAMRA-3′	99073–99091	Real-time PCR		
U23F	23S rRNA	5′-GATGCCTTGGCATTGATAGGCGATGAAGGA-3′	98502–98531	PCR/sequencing	602	Everett *et al.* (1999a)
23SIGR		5′-TGGCTCATCATGCAAAAGGCA-3′	99083–99103	PCR/sequencing		
CprpoBFc	*rpoB*	5′-CGAACTTGTAGAAGAAGCTGTTCA-3′	94409–94432	PCR/sequencing	733	This study
CprpoBRc		5′-AAAGCGTTCACCTGTCTTCC-3′	95122–95141	PCR/sequencing		
OUT1	*ygeD-urk* spacer	5′-GTTAGGGTGGTTTTCCCAGC-3′	221724–221743	PCR/sequencing	621	Cochrane *et al.* (2005)
OUT2		5′-GAGATAACGATTCTGAGGCC-3′	222325–222344	PCR/sequencing		

FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

1999) and compared to the *Chlamydiales* sequences of 16S rRNA, 23S rRNA, *rpoB* and *ompA* genes available in GenBank, including three biovars of *C. pneumoniae*. *C. pneumoniae* isolates whose sequences were used for comparative analysis are shown in Table 2. ClustalW multiple sequence alignment was performed using the MegAlign 5.0 program (DNAStar). The sequences of the entire *ompA* and 16S rRNA genes, partial sequences of 23S rRNA and *rpoB* genes and the *ygeD–urk* intergenic spacer from bandicoot *C. pneumoniae* were submitted to GenBank with the accession numbers DQ358972, DQ444323, DQ465990, DQ460031 and DQ463439, respectively.

RESULTS AND DISCUSSION

This is believed to be the first report of *C. pneumoniae* isolated and characterized from a third affected mammalian species. This species, WBBs, are small endangered marsupials whose natural habitat is currently limited to the Bernier and Dorre Islands off the West Coast of Australia. However, uncultured unspeciated *Chlamydiales* and *Chlamydophila pecorum* have been previously detected in these animals by molecular-based methods (Bodetti *et al.*, 2003; Warren *et al.*, 2005).

Isolation and speciation

Ten swab specimens (six ocular, three throat and one nasal) from nine WBBs were positive by isolation in cell culture followed by staining with a family-specific mAb against chlamydial lipopolysaccharide. These isolates were propagated by cell culture to 10^3–10^4 inclusion forming units ml^{-1} and were confirmed as *Chlamydiaceae* by 23S rRNA gene-based PCR. Sequence analysis of the 16S and 23S rRNA signature sequences described by Everett *et al.* (1999a) revealed that all 10 bandicoot isolates belonged to the *C. pneumoniae* species and were more than 99.1 % similar to *C. pneumoniae* isolates of human and animal origin, with only 1–5 bp variations.

ompA gene-based PCR and sequencing

All ten bandicoot isolates tested positive by *C. pneumoniae*-specific *ompA*-based, PCR using the originally described protocol (Apfalter *et al.*, 2003), which confirmed them as *C. pneumoniae*. However, the fluorescence signal was approximately 1.7 times lower than in the human *C. pneumoniae* control as shown in Fig. 1. An increase in annealing temperature resulted in a negative fluorescence signal for all bandicoot isolates but did not affect the signal for the human *C. pneumoniae* control (data not shown). Possible sequence variation between bandicoot and human isolates in the area where published primers and probe are meant to bind the target sequence was suspected.

To address this performance problem of the *ompA*-based PCR, the entire o*mpA* gene was amplified and sequenced for all isolates. *ompA* genes of all bandicoot *C. pneumoniae* isolates were identical to each other. As seen in Fig. 2, nucleotide alignment of the 85 bp target region used in the *ompA*-based TaqMan assay did reveal one single nucleotide polymorphism (SNP) in the forward primer and two SNPs in the probe sequence as compared to human isolates of *C. pneumoniae*. Overall, the entire *ompA* gene sequence from bandicoot *C. pneumoniae* was found to be 98.2 and 94.6 % similar to human and equine biovars, respectively (Table 3), and almost identical, 99.9 % similarity, to koala biovar (1 bp difference). This SNP at position 982 resulted in an amino acid substitution from alanine in the koala biovar to proline in the bandicoot *C. pneumoniae*.

16S rRNA gene sequencing

16S rRNA gene, which is also frequently used as a target for identification and speciation of *Chlamydiales* and other bacteria, was also amplified and sequenced for all bandicoot isolates. All bandicoot isolates were identical to each other and very similar to human and animal *C. pneumoniae* isolates as shown in Table 3. Minor nucleotide differences in both 16S rRNA and *ompA* genes may indicate genomic diversity within animal *C. pneumoniae* strains, as suggested by Hotzel *et al.* (2001).

The identification of *C. pneumoniae* infection in WBBs further proves that this species is not restricted to humans, and may exist in an animal reservoir with a possible transmission risk to humans. Coles *et al.* (2001) demonstrated that animal strains of koala biovar are able to infect and multiply in human respiratory cells and monocytes. Recently, Cochrane *et al.* (2005) identified an animal genotype of *C. pneumoniae* in carotid arteries and peripheral blood mononuclear cell (PBMC) specimens from humans, and human genotype in PBMC specimens from koalas by using nested *ompA*- and *ygeD–urk*-based PCR and sequencing assays. The authors suggested that *C. pneumoniae* might be capable of being transmitted between human and animals.

Genotyping

Data on the genotyping of human and animal strains of *C. pneumoniae* are limited. For genotyping purposes three genomic targets were selected: variable domain 4 region (VD4) of the *ompA* gene, the *ygeD–urk* intergenic spacer and a region of the *rpoB* gene matching a *C. pneumoniae*-specific *Pst*I fragment.

VD4 genotyping

VD4 was proposed as a possible genotyping target due to its small size and high degree of variability between human and animal isolates (Cochrane *et al.*, 2005; Wardrop *et al.*, 1999). Bodetti *et al.* (2002) suggested a genotyping system based on as little as 1 bp sequence polymorphism in the VD4 segment of the *ompA* gene. We analysed *C. pneumoniae* VD4 sequences publicly available in the GenBank database and identified 22 sequences, which we separated into 3 human and 7 animal genotype groups (based on their sequence identity within a group) as presented in Table 4. Sequence alignment of the 174 bp region of the VD4 segment demonstrated that bandicoot isolates differed from human isolates by 6–7 SNPs and were identical to previously reported koala and frog isolates (genotype group A5) and,

Table 2. *C. pneumoniae* sequences used for comparative analysis

C. pneumoniae **(host, isolate)**	**Gene**	**GenBank accession no.**	**Reference**
Human, TW-183	*ompA*	AE017159	–
	16S rRNA	AE017160	–
	23S rRNA	AE017160	–
	rpoB	AE017157	–
	ygeD–urk intergenic spacer	AE017159	–
Human, AR39	*ompA*	AE002161	Read *et al.* (2003)
	16S rRNA	AE002161	Read *et al.* (2003)
	23S rRNA	AE002161	Read *et al.* (2003)
	rpoB	AE002161	Read *et al.* (2003)
	ygeD–urk intergenic spacer	AE002161	Read *et al.* (2003)
Human, CWL029	*ompA*	AE001363	Kalman *et al.* (1999)
	16S rRNA	AE001363	Kalman *et al.* (1999)
	23S rRNA	AE001363	Kalman *et al.* (1999)
	rpoB	AE001363	Kalman *et al.* (1999)
	ygeD–urk intergenic spacer	AE001363	Kalman *et al.* (1999)
Human, J138	*ompA*	BA000008	Shirai *et al.* (2000)
	16S rRNA	BA000008	Shirai *et al.* (2000)
	23S rRNA	BA000008	Shirai *et al.* (2000)
	rpoB	BA000008	Shirai *et al.* (2000)
	ygeD–urk intergenic spacer	BA000008	Shirai *et al.* (2000)
Human, MS isolate	*ompA*	AF131889	Sriram *et al.* (1998)
Human, IOL 207	*ompA*	M64064	Carter *et al.* (1991)
	16S rRNA	Z49874	Wilson *et al.* (1996)
Human, LKK-1	*ompA*	AY555078	–
Human, CpnB	*ompA*	AY426606	Cochrane *et al.* (2005)
Human, CpnC	*ompA*	AY426607	Cochrane *et al.* (2005)
Human, CpnIII	*ygeD–urk* intergenic spacer	AY427827	Cochrane *et al.* (2005)
Western barred bandicoot, WBB	*ompA*	DQ358972	This study
	16S rRNA	DQ444323	This study
	23S rRNA	DQ465990	This study
	rpoB	DQ460031	This study
	ygeD–urk intergenic spacer	DQ463439	This study
African clawed frog, CPXT1	*ompA*	AF184214	Reed *et al.* (2000)
	16S rRNA	AF139200	Reed *et al.* (2000)
African frog, DE177	*ompA*	AF347608	Hotzel *et al.* (2001)
	16S rRNA	AF347610	Hotzel *et al.* (2001)
Blue Mountains tree frog, tree frog type I	*ompA*	AY026516	Bodetti *et al.* (2002)
Giant barred frog, frog Mi-1	*ompA*	AF102830	Berger *et al.* (1999)
Burmese python, Burmese python type I	*ompA*	AY026517	Bodetti *et al.* (2002)
Iguana, iguana type I	*ompA*	AY026518	Bodetti *et al.* (2002)
	16S rRNA	AF451290	Bodetti *et al.* (2002)
Puff adder, puff adder type I	*ompA*	AY026519	Bodetti *et al.* (2002)
Green sea turtle, turtle type I	*ompA*	AY026520	Bodetti *et al.* (2002)
Chameleon, chameleon type I	*ompA*	AY026515	Bodetti *et al.* (2002)
Koala, koala type I	*ompA*	M73038	Kaltenboeck *et al.* (1993)
Koala, LPConU	*ompA*	AF100958	Wardrop *et al.* (1999)
	16S rRNA	AF100957	Wardrop *et al.* (1999)
Koala, koala	*ompA*	X72023	Girjes *et al.* (1994)
Horse, N16	*ompA*	L04982	Storey *et al.* (1993)
	16S rRNA	U68426	Everett *et al.* (1999a)
	23S rRNA	U68426	Everett *et al.* (1999a)

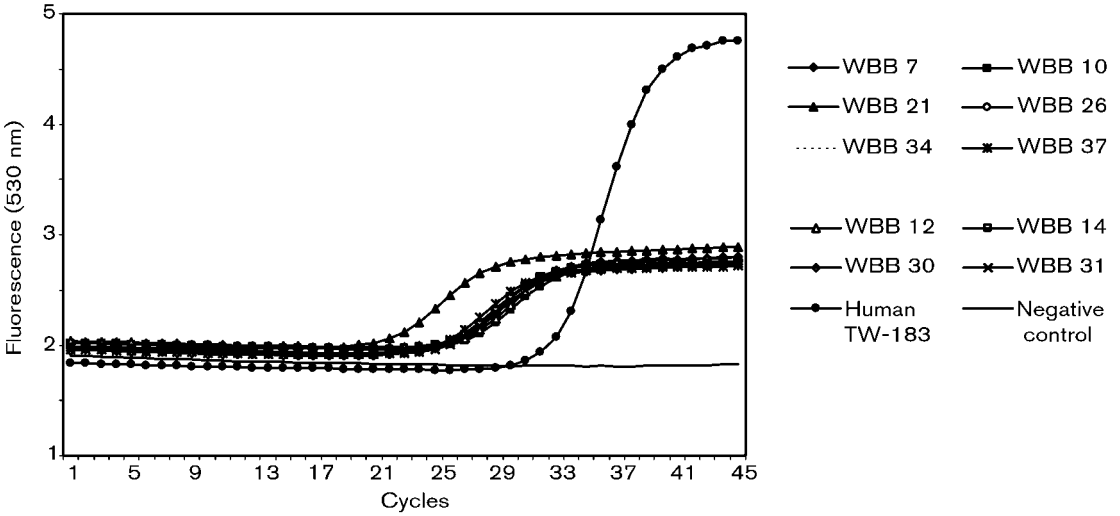

Fig. 1. Fluorescence signal of species-specific *ompA*-based real-time PCR of bandicoot *C. pneumoniae* isolates (WBB).

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272                                                                    356
........A.....................G.................T.......................................... WBB
GATCCGCTGCTGCAAACTATACTACTGCCGTAGATAGACCTAACCCGGCCTACAATAAGCATTTACACGATGCAGAGTGGTTCAC Human
Primer QMOMP1              Probe QMOMPS                          Primer QMOMP2
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Fig. 2. Nucleotide sequence alignment of the 85 bp *ompA* target region used in the *C. pneumoniae*-specific TaqMan PCR assay. Dots indicate a nucleotide is the same as in sequences of human isolates (TW-183, CWL029, AR-39 and J138).

Table 3. Sequence similarities (%) in five genomic regions of WBB isolates compared to existing *C. pneumoniae* biovars/isolates.

C. pneumoniae biovar	*ompA* gene (1170 bp)	16S rRNA gene (1552 bp)	23S rRNA segment (551 bp)	*rpoB* segment (687 bp)	*ygeD–urk* intergenic spacer (320 bp)
Human biovar					
TW-183	98.2	99.6	99.6	99.9	99.1
AR39	98.2	99.6	99.6	99.9	99.1
J138	98.2	99.4	99.6	99.9	99.1
CWL029	98.2	99.5	99.6	99.9	95.9
IOL-207	98.2	99.6	NA	NA	NA
Koala biovar					
Koala type I, LpConU	99.9	99.5	NA	NA	NA
Equine biovar					
N16	94.6	99.3	99.1	NA	NA
Other isolates					
African clawed frog	NA	99.7	NA	NA	NA
Other *Chlamydophila* species	⩽74.6	⩽96.3	⩽94.7	⩽84.0	–*

NA, Not available.

*No significant similarity found by BLASTN search.

Table 4. *C. pneumoniae* genotype groups based on the bp difference at the VD4 locus of the *ompA* gene

Origin	Genotype group	*C. pneumoniae* isolates
Human	H1	TW-183, AR39, CWL029, J138, MS isolate, IOL 207, LKK-1
	H2	CpnB
	H3	CpnB
Animal	A1	Burmese python type I, iguana type I, puff adder type I, turtle type I, tree frog type I
	A2	Chameleon type I
	A3	DE177
	A4	CPXT1
	A5	Koala type I, LPConU, frog Mi-1, WBB isolates
	A6	Koala
	A7	N16

according to the Cochrane *et al.* (2005) designations, would be assigned to genotype D. Interestingly, the animal *C. pneumoniae* isolates from genotype group A1 from reptiles and amphibians (Bodetti *et al.*, 2002) were absolutely identical to human isolates from group H1 as shown in Fig. 3.

ygeD–urk genotyping

The intergenic spacer region between *ygeD* and *urk* genes differs by the orientation of a 23 bp segment in one of the human *C. pneumoniae* strains (Read *et al.*, 2000) and was used for genotyping of *C. pneumoniae* (Cochrane *et al.*, 2005). The results of the 320 bp *ygeD–urk* spacer genotyping are shown in Fig. 4. The bandicoot isolates were identical to CpnIII genotype recently found in humans (Cochrane *et al.*, 2005) and had the 23 bp invertible region in the same orientation as human TW-183, AR39 and J138 strains. There was a 3 bp difference as compared to human isolates.

rpoB genotyping

rpoB gene was of relevant interest because of its current use as a target in *C. pneumoniae*-specific PCR assays (Dowell *et al.*, 2001; Loens *et al.*, 2006). Sequence analysis of the *rpoB* gene region that matched a *C. pneumoniae*-specific *Pst*I fragment (Campbell *et al.*, 1992) showed a 1 bp polymorphism as compared to human strains of *C. pneumoniae* (Fig. 5). This nucleotide change at position 2946 was a silent mutation with no amino acid change (alanine).

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826                                                                                                912
..T.......................................................................................... WBB
TACAGACTAAACTCTTTAGTGCCATACATTGGAGTACAATGGTCTCGAGCAACTTTTGATGCTGATAACATCCGCATTGCTCAGCCA Group H1
.......................................................................................... Group H2
.......................................................................................... Group H3
.......................................................................................... Group A1
.......................................................................................... Group A2
.......................................................................................... Group A3
.....G.....................................................................................  Group A4
..T....................................................................................... Group A5
..T....................................................................................... Group A6
..T.....C.........................C..................................................... Group A7

913                                                                                                999
.....................................................A.G...A.....C......G.......... WBB
AAACTACCTACAGCTGTTTTAAACTTAACTGCATGGAACCCTTCTTTACTAGGAAATGCCACAGCATTGTCTACTACTGATTCGTTC Group H1
.........................................C.............................................. Group H2
.....................................................G.................................. Group H3
.......................................................................................... Group A1
............T.............................................................................. Group A2
.....................................................A.....C......A.......... Group A3
.....................................................A............A.......... Group A4
.....................................................A.G...A.....C......G.......... Group A5
.....................................................A.G...A.....G......G.......... Group A6
..G...........A.....................................G.G.........TG.T...T.AT.....CAA... Group A7
```

Fig. 3. Nucleotide sequence alignment of a 174 bp region within the VD4 segment of bandicoot *C. pneumoniae ompA* gene with human and animal genotype. Dots indicate a nucleotide is the same as in sequences of human H1 group. The GenBank accession no. for WBB isolates is DQ358972.

Fig. 4. Nucleotide sequence alignment of a 320 bp *ygeD–urk* intergenic spacer of bandicoot and human isolates. Dots indicate a nucleotide is the same as in sequences of human AR39, TW-183 and J138 isolates, and dashes represent gaps in the sequences. CpnIII is a *C. pneumoniae* genotype identified in human carotid specimens, GenBank accession no. AY427827 (Cochrane *et al.*, 2005).

C. pneumoniae species-specific PCR assays and sequence polymorphism

The results of this study may have important implications for molecular diagnosis of *C. pneumoniae* infection in both animals and humans. Since most of the *C. pneumoniae*-specific PCR assays were developed and evaluated using human isolates, they may not be appropriate for detection of animal strains. Even minor sequence variability in the target regions can significantly compromise the sensitivity of the

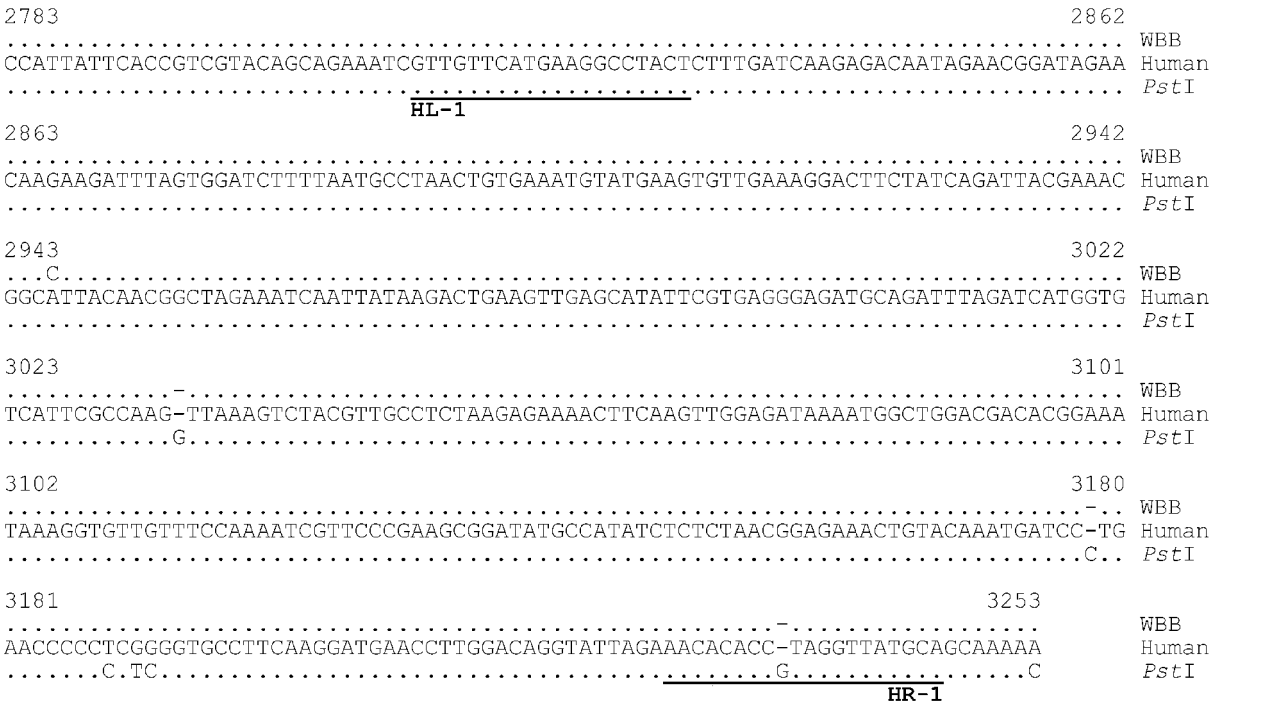

Fig. 5. Nucleotide sequence alignment of a 474 bp region of *rpoB* gene (matching a *C. pneumoniae*-specific *Pst*I fragment) of human and WBB isolates. Dots indicate a nucleotide is the same as in human isolates, represented by TW-183, CWL029, AR39 and J138, and dashes represent gaps in the sequence. Primer sequences HL-1 and HR-1 used in the *C. pneumoniae*-specific PCR assay (Campbell *et al.*, 1992) are shown in bold/underlined.

Table 5. Sequence variability in the target regions of the selected species-specific PCR assays as compared to bandicoot *C. pneumoniae* isolates

PCR type	Gene, product size (bp)	Sequence polymorphism (bp)				Reference
		Forward primer (outer/inner)	Reverse primer (outer/inner)	Probe	Target total	
Real-time	*ompA*, 185	1	0	2	3	Apfalter *et al.* (2003)
Real-time	*ompA*, 134	0	0	0	1	Meijer *et al.* (1998)
Real-time	*ompA*, 79	0	0	0	0	Kuoppa *et al.* (2002)
Real-time	*ompA*, 109	0	0	2	5	Tondella *et al.* (2002)
Real-time	*ompA*, 126	0	0	0	5	Tondella *et al.* (2002)
Real-time	*ompA*, 72	0	0	0	0	Kohlhepp *et al.* (2005)
Nested*	*ompA*, 333	0/0	0/1	–	4	Tong & Sillis (1993)
Nested	*ompA*, 497	1/1	0/1	–	13	Lindholt *et al.* (1998)
Single-step*	*rpoB*, 438	0	1	–	3	Campbell *et al.* (1992)
Real-time	*rpoB*, 82	0	0	0	0	Welti *et al.* (2003)
Real-time	16S rRNA, 149	0	0	0	2	Meijer *et al.* (1998)
Single-step*	16S rRNA, 195	0	0	–	0	Madico *et al.* (2000)
Real-time	16S rRNA, 195	0	0	0	2	Hardick *et al.* (2004)
Single-step*	16S rRNA, 464	0	0	–	2	Gaydos *et al.* (1992)
Real-time	16S rRNA, 154	1	0	0	2	Templeton *et al.* (2005)

*These assays meet CDC validation criteria (Dowell *et al.*, 2001).

PCR assay and result in false negative results due to either target amplification failure (primer sequence polymorphism) or target detection failure (probe sequence polymorphism) (Kwok *et al.*, 1990; Stevenson *et al.*, 2005). As we demonstrated in this study a real-time *ompA*-based PCR assay developed for human strains either generated a lower fluorescent signal or failed to detect bandicoot *C. pneumoniae* isolates because there was one SNP in the forward primer and there were two SNPs in the probe. Similar assay-performance problems due to target-sequence variability have been recently reported for real-time PCRs for the detection of herpes simplex virus and *Listeria monocytogenes* (Rodríguez-Lázaro *et al.*, 2004; Stevenson *et al.*, 2005). We analysed several published and currently used *C. pneumoniae* species-specific PCR assays for the presence of the sequence polymorphism in the target regions as shown in Table 5. We found that 6 out of 15 species-specific PCR assays had sequence variability in primer and/or probe that may affect their performance in detecting bandicoot strain of *C. pneumoniae*, and possibly other animal and human strains where minor sequence polymorphism may be present.

The expanding number of animal hosts and isolates of *C. pneumoniae*, and genetic similarity of animal and human strains raises some interesting questions about the evolution and epidemiology of this pathogen. It is quite possible that *C. pneumoniae* was primarily an animal pathogen, which was only recently, in evolutionary terms, acquired by humans. Considering almost universal exposure of humans to *C. pneumoniae* and generally inefficient mode of transmission from person to person (Blasi *et al.*, 1998), one could hypothesize that an environmental source of *C. pneumoniae* should be in close proximity to humans. While possible zoonotic transmission of *C. pneumoniae* from exotic animals occupying a unique environmental niche (koalas and bandicoots) to humans seems unlikely, acquisition by contact with animals living in urban or rural areas, including domestic and production animals, either directly or via a transmission vector such as free-living protozoa, could be possible (Essig *et al.*, 1997). However, in addition to the previously described equine biovar (Storey *et al.*, 1993) there have been only two reports of possible *C. pneumoniae* infection in domesticated animals. Sako *et al.* (2002) described detection of *C. pneumoniae* antigens in vascular specimens from dogs and Canderle *et al.* (2005) reported high prevalence of *C. pneumoniae* species-specific antibodies in boars.

More targeted research efforts are necessary to determine the prevalence and host range of *C. pneumoniae* in environmental reservoirs of different geographical regions since most of the published reports on animal strains were simply serendipitous findings. There is also a need for the development of relevant and consistent genotyping system to be used for epidemiological surveillance, pathogenesis, evolution studies and characterization of novel *C. pneumoniae* isolates.

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REFERENCES

Apfalter, P., Barousch, W., Nehr, M., Makristathis, A., Willinger, B., Rotter, M. & Hirschl, A. M. (2003). Comparison of a new quantitative *ompA*-based real-time PCR TaqMan assay for detection of *Chlamydia pneumoniae* DNA in respiratory specimens with four conventional PCR assays. *J Clin Microbiol* **41**, 592–600.

Balin, B. J., Gerard, H. C., Arking, E. J., Appelt, D. M., Branigan, P. J., Abrams, J. T., Whittum-Hudson, J. A. & Hudson, A. P. (1998). Identification and localization of *Chlamydia pneumoniae* in the Alzheimer's brain. *Med Microbiol Immunol* **187**, 23–42.

Berger, L., Volp, K., Mathews, S., Speare, R. & Timms, P. (1999). *Chlamydia pneumoniae* in a free-ranging giant barred frog (*Mixophyes iteratus*) from Australia. *J Clin Microbiol* **37**, 2378–2380.

Blasi, F., Tarsia, P., Arosio, C., Fagetti, L. & Allegra, L. (1998). Epidemiology of *Chlamydia pneumoniae*. *Clin Microbiol Infect* **4** (Suppl.), S1–S6.

Blasi, F., Damato, S., Cosentini, R., Tarsia, P., Raccanelli, R., Centanni, S., Allegra, L. & the *Chlamydia* InterAction with C.O. P. D. (C. I. A. C.) Study Group (2002). *Chlamydia pneumoniae* and chronic bronchitis: association with severity and bacterial clearance following treatment. *Thorax* **57**, 672–676.

Bodetti, T. J., Jacobson, E., Wan, C., Hafner, L., Pospischil, A., Rose, K. & Timms, P. (2002). Molecular evidence to support the expansion of the hostrange of *Chlamydophila pneumoniae* to include reptiles as well as humans, horses, koalas and amphibians. *Syst Appl Microbiol* **25**, 146–152.

Bodetti, T. J., Viggers, K., Warren, K., Swan, R., Conaghty, S., Sims, C. & Timms, P. (2003). Wide range of *Chlamydiales* types detected in native Australian mammals. *Vet Microbiol* **96**, 177–187.

Campbell, L. A., Melgosa, M. P., Hamilton, D. J., Kuo, C.-C. & Grayston, J. T. (1992). Detection of *Chlamydia pneumoniae* by polymerase chain reaction. *J Clin Microbiol* **30**, 434–439.

Canderle, J., Pospisil, L., Stroblova, H. & Veznik, Z. (2005). *Chlamydophila pneumoniae* antibodies in swine. *Acta Vet* **74**, 81–86.

Carter, M. W., al-Mahdawi, S. A., Giles, I. G., Treharne, J. D., Ward, M. E. & Clark, I. N. (1991). Nucleotide sequence and taxonomic value of the major outer membrane protein gene of *Chlamydia pneumoniae* IOL-207. *J Gen Microbiol* **137**, 465–475.

Cochrane, M., Walker, P., Gibbs, H. & Timms, P. (2005). Multiple genotypes of *Chlamydia pneumoniae* identified in human carotid plaque. *Microbiology* **151**, 2285–2290.

Coles, K. A., Timms, P. & Smith, D. W. (2001). Koala biovar of *Chlamydia pneumoniae* infects human and koala monocytes and induces increased uptake of lipids *in vitro*. *Infect Immun* **69**, 7894–7897.

Dowell, S. F., Peeling, R. W., Boman, J., Carlone, G. M., Fields, B. S., Guarner, J., Hammerschlag, M. R., Jackson, L. A., Kuo, C.-C. & other authors (2001). Standardizing *Chlamydia pneumoniae* assays: recommendations from the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada). *Clin Infect Dis* **33**, 492–503.

Essig, A., Heinemann, M., Simnacher, U. & Marre, R. (1997). Infection of *Acanthamoeba castellanii* by *Chlamydia pneumoniae*. *Appl Environ Microbiol* **63**, 1396–1399.

Everett, K. D., Bush, R. M. & Andersen, A. A. (1999a). Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst Bacteriol* **49**, 415–440.

Everett, K. D., Hornung, L. J. & Andersen, A. A. (1999b). Rapid detection of the *Chlamydiaceae* and other families in the order *Chlamydiales*: three PCR tests. *J Clin Microbiol* **37**, 575–580.

Gaydos, C. A., Quinn, T. C. & Eiden, J. J. (1992). Identification of *Chlamydia pneumoniae* by DNA amplification of the 16S rRNA gene. *J Clin Microbiol* **30**, 796–800.

Girjes, A. A., Carrick, F. N. & Lavin, M. F. (1994). Remarkable sequence relatedness in the DNA encoding the major outer membrane protein of *Chlamydia psittaci* (koala type I) and *Chlamydia pneumoniae*. *Gene* **138**, 139–142.

Hahn, D. L., Dodge, R. W. & Golubjatnikov, R. (1991). Association of *Chlamydia pneumoniae* (strain TWAR) infection with wheezing, asthmatic bronchitis, and adult-onset asthma. *JAMA* **266**, 225–230.

Hardick, J., Maldeis, N., Theodore, M., Wood, B. J., Yang, S., Lin, S., Quinn, T. & Gaydos, C. (2004). Real-time PCR for *Chlamydia pneumoniae* utilizing the Roche Lightcycler and a 16S rRNA gene target. *J Mol Diagn* **6**, 132–136.

Hotzel, H., Grossmann, E., Mutschmann, F. & Sachse, K. (2001). Genetic characterization of a *Chlamydophila pneumoniae* isolate from an African frog and comparison to currently accepted biovars. *Syst Appl Microbiol* **24**, 63–66.

Jacobson, E. R., Heard, D. & Andersen, A. (2004). Identification of *Chlamydophila pneumoniae* in an emerald tree boa, *Corallus caninus*. *J Vet Diagn Invest* **16**, 153–154.

Kalman, S., Mitchell, W., Marathe, R., Lammel, C., Fan, J., Hyman, R. W., Olinger, L., Grimwood, J., Davis, R. W. & Stephens, R. (1999). Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. *Nat Genet* **21**, 385–389.

Kaltenboeck, B., Kousoulas, K. G. & Storz, J. (1993). Structures of and allelic diversity and relationships among the major outer membrane protein (*ompA*) genes of the four chlamydial species. *J Bacteriol* **175**, 487–502.

Kohlhepp, S. J., Hardick, J. & Gaydos, C. (2005). *Chlamydia pneumoniae* in peripheral blood mononuclear cells isolated from individuals younger than 20 years or older than 60 years. *J Clin Microbiol* **43**, 3030.

Kuoppa, Y., Boman, J., Scott, L., Kumlin, U., Eriksson, I. & Allard, A. (2002). Quantitative detection of respiratory *Chlamydia pneumoniae* infection by real-time PCR. *J Clin Microbiol* **40**, 2273–2274.

Kwok, S., Kellogg, D. E., McKinney, N., Spasic, D., Goda, L., Levenson, C. & Sninsky, J. J. (1990). Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. *Nucleic Acids Res* **18**, 999–1005.

Lindholt, J. S., Ostergard, L., Henneberg, E. W., Fasting, H. & Andersen, P. (1998). Failure to demonstrate *Chlamydia pneumoniae* in symptomatic abdominal aortic aneurysms by a nested polymerase chain reaction (PCR). *Eur J Vasc Endovasc Surg* **15**, 161–164.

Loens, K., Beck, T., Ursi, D., Pattyn, S., Goossens, H. & Ieven, M. (2006). Two quality control exercises involving nucleic acid amplification methods for detection of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* and carried out 2 years apart (in 2002 and 2004). *J Clin Microbiol* **44**, 899–908.

Madico, G. E., Quinn, T. C., Boman, J. & Gaydos, C. A. (2000). Touchdown enzyme time release PCR for detection and identification of *Chlamydia tracomatis, C. pneumoniae*, and *C. psittaci* using the 16S and 16S–23S spacer rRNA genes. *J Clin Microbiol* **38**, 1085–1093.

Meijer, A., van der Vliet, J. A., Schouls, L. M., de Vries, A., Roholl, P. J. & Ossewaarde, J. M. (1998). Detection of microorganisms in vessel wall specimens of the abdominal aorta: development of a PCR assay in the absence of a gold standard. *Res Microbiol* **149**, 577–583.

Peeling, R. W. & Brunham, R. C. (1996). *Chlamydiae* as pathogens: new species and new issues. *Emerg Infect Dis* **2**, 307–319.

Read, T. D., Brunham, R. C., Shen, C., Gill, S. R., Heidelberg, J. F., White, O., Hickey, E. K., Peterson, J., Utterback, T. & other authors

(2000). Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. *Nucleic Acids Res* **28**, 1397–1406.

Reed, K. D., Ruth, G. R., Meyer, J. A. & Shukla, S. K. (2000). *Chlamydia pneumoniae* infection in a breeding colony of African clawed frogs (*Xenopus tropicalis*). *Emerg Infect Dis* **6**, 196–199.

Roblin, P. M., Dumornay, W. & Hammerschlag, M. R. (1992). Use of HEp-2 cells for improved isolation and passage of *Chlamydia pneumoniae*. *J Clin Microbiol* **30**, 1968–1971.

Rodríguez-Lázaro, D., Hernández, M., Scortti, M., Esteve, T., Vázquez-Bolan, J. A. & Pla, M. (2004). Quantitative detection of *Listeria monocytogenes* and *Listeria innocua* by real-time PCR: assessment of *hly*, *iap*, and *lin02483* targets and AmpliFluor technology. *Appl Environ Microbiol* **70**, 1366–1377.

Rozen, S. & Skaletsky, H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, pp. 365–386. Edited by S. Krawetz & S. Misener. Totowa, NJ: Humana Press.

Sako, T., Takahashi, T., Takehana, K., Uchida, E., Nakade, T., Umemura, T. & Taniyama, H. (2002). Chlamydial infection in canine atherosclerotic lesions. *Atherosclerosis* **162**, 253–259.

Shirai, M., Hirakawa, H., Kimoto, M., Tabuchi, M., Kishi, F., Ouchi, K., Shiba, T., Ishii, K., Hattori, M. & other authors (2000). Comparison of whole genome sequences of *Chlamydia pneumoniae* J138 from Japan and CWL029 from USA . *Nucleic Acids Res* **28**, 2311–2314.

Sriram, S., Mitchell, W. & Stratton, C. (1998). Multiple sclerosis associated with *Chlamydia pneumoniae* infection of the CNS. *Neurology* **50**, 571–572.

Stevenson, J., Hymas, W. & Hillyard, D. (2005). Effect of sequence polymorphisms on performance of two real-time PCR assays for detection of herpes simplex virus. *J Clin Microbiol* **43**, 2391–2398.

Storey, C., Lusher, M., Yates, P. & Richmond, S. (1993). Evidence for *Chlamydia pneumoniae* of non-human origin. *J Gen Microbiol* **139**, 2621–2626.

Tatusova, T. A. & Madden, T. L. (1999). Blast 2 sequences – a new tool for comparing protein and nucleotide sequences. *FEMS Microbiol Lett* **174**, 247–250.

Templeton, K. E., Scheltingha, S. A., Van den Eeden, W. C. J. F. M., Graffelman, A. W., van den Broek, P. H. & Claas, E. (2005). Improved diagnosis of etiology of community-acquired pneumonia using real-time PCR. *Clin Infect Dis* **41**, 345–351.

Tondella, M. L., Talkington, D. F., Holloway, B. P., Dowell, S. F., Cowley, K., Soriano-Gabarro, M., Elkind, M. S. & Fields, B. S. (2002). Development and evaluation of real-time PCR-based fluorescence assays for detection of *Chlamydia pneumoniae*. *J Clin Microbiol* **40**, 575–583.

Tong, C. Y. & Sillis, M. (1993). Detection of *Chlamydia pneumoniae* and *Chlamydia psittaci* in sputum samples by PCR. *J Clin Pathol* **46**, 313–317.

Wardrop, S., Fowler, A. O'Callaghan P., Giffard, P. & Timms, P. (1999). Characterization of the koala biovar of *Chlamydia pneumoniae* at four gene loci – *ompAVD4*, *ompB*, 16S rRNA, *groESL* spacer region. *Syst Appl Microbiol* **22**, 22–27.

Warren, K., Swan, R., Bodetti, T., Friend, T., Hill, S. & Timms, P. (2005). Ocular *Chlamydiales* infections of Western barred bandicoots (*Perameles bougainville*) in Western Australia. *J Zoo Wildl Med* **36**, 100–102.

Welti, M., Jaton, K., Altwegg, M., Sahli, R., Wenger, A. & Bille, J. (2003). Development of a multiplex real-time quantitative PCR assay to detect *Chlamydia pneumoniae*, *Legionella pneumophila*, and *Mycoplasma pneumoniae* in respiratory tract secretions. *Diagn Microbiol Infect Dis* **45**, 85–95.

Wilson, P. A., Phipps, J., Samuel, D. & Saunders, N. A. (1996). Development of a simplified polymerase chain reaction-enzyme immunoassay for the detection of *Chlamydia pneumoniae*. *J Appl Bacteriol* **80**, 431–438.

Wong, Y. K., Gallagher, P. J. & Ward, M. E. (1999). *Chlamydia pneumoniae* and atherosclerosis. *Heart* **81**, 232–223.