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2
3 **Title:** MOLECULAR CHARACTERIZATION OF *CHLAMYDOPHILA PNEUMONIAE* ISOLATES
4 FROM WESTERN BARRED BANDICOOTS

5
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18
19 **Running title:** novel animal strain of *C. pneumoniae*

20
21 **Subject category:** Veterinary microbiology, Diagnostics, typing and identification

22
23 **Abbreviations:** WBB, western barred bandicoots

24 **Footnote:** The GenBank accession numbers for the *ompA* and *16S rRNA* genes, partial sequences of *23S*
25 *rRNA* and *rpoB* genes and *YgeD-urk* intergenic spacer from bandicoot *C. pneumoniae* isolates are
26 DQ358972, DQ444323, DQ465990, DQ460031 and DQ463439, respectively.

27 SUMMARY

28

29 *Chlamydophila pneumoniae* is an obligate intracellular respiratory pathogen that has been associated with
30 pneumonia and chronic bronchitis, atherosclerosis, asthma and other chronic diseases in humans.

31 However, *C. pneumoniae* is not restricted to humans, as originally thought, and can cause infections in
32 several animal hosts. We isolated *C. pneumoniae* in cell culture from nine western barred bandicoots
33 (*Perameles bougainville*) from Australia. The sequences of five genomic regions were determined
34 including full-length sequences of the *16S rRNA*, *ompA* genes and *YgeD-urk* intergenic spacer and partial
35 sequences of the *23S rRNA*, and *rpoB* genes. Sequence analysis of the entire *16S rRNA* and *ompA* genes
36 from bandicoot isolates demonstrated that they were 98.2- 98.3% similar to human isolates, 94.6-99.3%
37 to the equine biovar and almost identical with 99.5-99.9% similarity to the koala biovar. Comparative
38 genotyping of the variable domain 4 region of the *ompA* gene demonstrated that bandicoot isolates
39 seemed to be identical to the animal genotype that has been recently identified in human carotid plaque
40 specimens. Minor sequence polymorphism observed in *ompA*, *16S rRNA* and *rpoB* genes of animal
41 isolates, indicating genomic diversity within *C. pneumoniae*, may have important implications for
42 diagnostic PCR assays leading to false negative results. Forty percent of selected previously published
43 species-specific PCR assays were found to have sequence variability in primer and/or probe that may
44 affect their performance in detecting bandicoot isolates of *C. pneumoniae*, or possibly other animal and
45 human strains where minor sequence polymorphisms may be present. The data of this study support the
46 previous observations that *C. pneumoniae* is not restricted to humans and may be widespread in an animal
47 reservoir with a potential risk of possible transmission to humans.

48

49 INTRODUCTION

50

51 *Chlamydophila pneumoniae* is an obligate intracellular bacterium responsible for respiratory infections
52 (pneumonia and bronchitis) in adults and children, affecting up to 70% of the population worldwide at
53 least once during his or her lifetime (Peeling & Brunham, 1996). Persistent *C. pneumoniae* infections

54 have been implicated in the development of atherosclerosis, asthma and other chronic diseases in humans
55 (Balin *et al.*, 1998; Blasi *et al.*, 2002; Hanh *et al.*, 1991; Wong *et al.*, 1999).

56
57 *C. pneumoniae* was initially thought to be an exclusively human pathogen. However, several studies
58 demonstrated that *C. pneumoniae* could also cause ocular, respiratory and urogenital infections in a wide
59 variety of animal species, including koalas, horses, frogs and reptiles (Berger *et al.*, 1999; Bodetti *et al.*,
60 2002; Hotzel *et al.*, 2001; Jacobson *et al.*, 2004; Storey *et al.*, 1993). Current taxonomic classification
61 divides *C. pneumoniae* into three distinct biovars: human biovar TWAR, biovar koala and biovar equine
62 (Everett *et al.*, 1999a). The distinction was based primarily on comparative sequence analysis of the *16S*
63 *rRNA*, *23S rRNA* and *ompA* genes and biological characteristics. Human *C. pneumoniae* isolates were
64 found to be almost identical to each other, with only 0.1 % difference in *16S rRNA* gene and 0.4%
65 difference in the *ompA* gene. While some of the animal isolates are almost identical to human strains, the
66 others seem to be genetically more diverse, with up to 6% *ompA* gene dissimilarity (Bodetti *et al.*, 2002).

67
68 The existence of animal strains of *C. pneumoniae* raises the issue of possible transmission risk to humans.
69 A recent study by Cochrane *et al.* (2005) where animal genotypes of *C. pneumoniae* were identified in
70 human specimens and human genotypes were detected in koalas, supports the possibility of such
71 transmission. However, no cases of zoonotic *C. pneumoniae* infections in humans have been described so
72 far.

73
74 In this study, *C. pneumoniae* was isolated from western barred bandicoots (*Perameles bougainville*) in
75 cell culture and characterized by sequence comparison of five genomic regions relevant for molecular
76 diagnostics, including *16S rRNA*, *23S rRNA*, *ompA* and *rpoB* genes and *ygeD-urk* intergenic spacer. The
77 entire *ompA* and *16S rRNA* genes, that are frequently used in species-specific PCR-based assays (Dowell
78 *et al.*, 2001; Loens *et al.*, 2006) were sequenced and compared to the published sequences of human and
79 animal *C. pneumoniae* isolates. Genotyping of the bandicoot isolates was performed in the variable
80 domain 4 of the *ompA* gene, *rpoB* gene and *ygeD-urk* intergenic spacer loci.

81

82 **METHODS**

83

84 **Animals.** All western barred bandicoots sampled in this study were from wild populations from Bernier
85 island and Dryandra, Western Australia. Thirty-seven conjunctival, nasal, throat and cloacal swab
86 specimens from twenty-one animals with clinical signs of ocular and/or respiratory disease were
87 examined.

88

89 **Isolation and propagation of *Chlamydiales*.** Isolation and propagation of *Chlamydiales* was performed
90 by cell culture, as previously described (Roblin *et al.*, 1992). Briefly, swab specimens were inoculated
91 onto HEp-2 cell monolayers (ATCC CCL-23) by centrifugation at 1,700 x *g* for 1 hour. Infected
92 monolayers were then overlaid with Iscove's DMEM medium (Sigma-Aldrich, St. Luis, MO)
93 supplemented with 10% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA), 20mM L-glutamine
94 and 1 µg/mL of cycloheximide and incubated at 35°C for 72 hours. Up to 6 passages were performed for
95 each isolate. Following incubation, inoculated HEp-2 cells were fixed in ethanol and stained for specific
96 chlamydial inclusions with Pathfinder Chlamydia Confirmation System (BioRad, Hercules, CA) for 30
97 min at 37°C.

98

99 **DNA extraction.** DNA from the cultured *Chlamydiales* was extracted using DNeasy Tissue kit (Qiagen,
100 Valencia, CA) according to the manufacturer's protocol. Human *C. pneumoniae* CWL029 (ATCC VR-
101 1310) and TW-183 (ATCC VR-2282) grown in HEp-2 cells were used as positive control in PCR assays.

102

103 ***Chlamydiaceae* specific PCR TaqMan assay.** The TQF and TQR primers and probe targeting the
104 *Chlamydiaceae* specific region of 23S *rRNA* gene were as described by Everett *et al.* (1999b) (Table 1).
105 PCR was performed using LightCycler 2.0 (Roche, Indianapolis, IN) system with DNA Master HybProbe
106 kit (Roche, Indianapolis, IN), containing FastStart *Taq* DNA polymerase at the following conditions:
107 initial denaturation step at 95°C for 10 min, then 45 cycles of denaturation at 95°C for 5 s, annealing at

108 60°C for 10 s, and extension at 72°C for 10 s. Products amplification was analyzed by manufacturers'
109 supplied software.

110

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

115

116 ***16S* and *23S rRNA* genes signature sequencing.** Amplification/sequencing primers used for *16S rRNA*
117 gene and *23S rRNA* gene products were 16SIGF, 16SIGR, U23F and 23SIGR (Table 1). PCR was
118 performed using Qiagen ProofStart DNA polymerase kit (Qiagen, Valencia, CA), with initial denaturation
119 at 95°C for 5 min, then 45 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at
120 72°C for 60 s, with a final extension for 5 min at 72°C. Following amplification, the products were
121 separated by electrophoresis in 2% agarose E-gel (Invitrogen, Carlsbad, CA) and visualized by using
122 ultraviolet transilluminator. PCR products were purified with QIAquick PCR Purification kit (Qiagen,
123 Valencia, CA) and sequenced in both directions (GeneWiz, North Brunswick, NJ).

124

125 ***OmpA* gene sequencing.** DNA fragment containing the entire *ompA* gene was amplified with CpompA1F
126 and CpompA3R primers (Table 1). PCR was performed using ProofStart DNA polymerase kit, with
127 initial denaturation step at 95°C for 5 min, then 40 cycles of denaturation at 94°C for 30 s, annealing at
128 55°C for 30 s, extension at 72°C for 60 s, with a final extension for 5 min at 72°C. Following
129 electrophoresis in 2% agarose E-gel. PCR products were visualized by using ultraviolet transilluminator.
130 PCR products containing 1170 bp *ompA* gene were sequenced in both directions, with overlap, using the
131 sequencing primers shown in Table 1. These primers were designed using Primer3 software
132 [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi] (Rozen & Skaletsky, 2000).

133

134 ***16S rRNA* gene sequencing.** DNA region containing the entire *16S rRNA* gene was amplified with
135 Cp16SF and Cp16SRb primers (Table 1). PCR was performed using ProofStart DNA polymerase kit,
136 with initial denaturation step at 95°C for 5 min, then 45 cycles of denaturation at 94°C for 30 s, annealing
137 at 55°C for 30 s, extension at 72°C for 60 s, with a final extension for 5 min at 72°C. Following
138 electrophoresis in 2% agarose E-gel, PCR products were visualized and purified with QIAquick PCR
139 Purification kit. PCR products containing 1552 bp *16S rRNA* gene were sequenced in both directions,
140 with overlap, using the following primers: Cp16SF1, Cp16SR1, Cp16SF2, Cp16SR2, Cp16SF3,
141 Cp16SR3, Cp16SF4 and Cp16SR4 (Table 1). The above primers were designed using Primer3 program.

142

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

149

150 ***YgeD-urk* intergenic spacer sequencing.** A 621 bp fragment spanning *YgeD-urk* intergenic spacer was
151 amplified and sequenced using OUT1 and OUT2 primers (Table 1). PCR was performed using ProofStart
152 DNA polymerase kit at the above conditions. Prior to sequencing products were purified from 2% agarose
153 E-gels with QIAquick PCR Purification kit.

154

155 **Sequence analysis.** The sequences were analyzed using BLAST 2 [[http://www.ncbi.nlm.nih.gov/blast/](http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html)
156 [bl2seq/ bl2.html](http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html)] (Tatusova & Madden, 1999) and compared to the Chlamydiales sequences of *16S rRNA*,
157 *23S rRNA*, *rpoB* and *ompA* genes available in GenBank, including three biovars of *C. pneumoniae*. *C.*
158 *pneumoniae* isolates which sequences were used for comparative analysis, are shown in Table 2.

159 ClustalW multiple sequence alignment was performed using MegAlign 5.0 program (DNASar, Madison,
160 WI). The sequences of the entire *ompA* and *16S rRNA* genes, partial sequences of *23S rRNA* and *rpoB*

161 genes and *YgeD-urk* intergenic spacer from bandicoot *C. pneumoniae* were submitted to GenBank;
162 accession numbers DQ358972, DQ444323, DQ465990, DQ460031 and DQ463439, respectively.

163

164 **RESULTS AND DISCUSSION**

165

166 This is the first report of *C. pneumoniae* isolated and characterized in a third affected mammalian species.

167 This species, western barred bandicoots are small endangered marsupials whose natural habitat is

168 currently limited to Bernier and Dorre islands of the West Coast of Australia. However, uncultured

169 unspiciated *Chlamydiales* and *C. pecorum* have been previously detected in these animals by molecular

170 based methods (Bodetti *et al.*, 2003; Warren *et al.*, 2005).

171

172 **Isolation and speciation.** Ten swab specimens (6 ocular, 3 throat and 1 nasal) from nine western barred

173 bandicoots were positive by isolation in cell culture followed by staining with family specific monoclonal

174 antibody against chlamydial lipopolysaccharide. These isolates were propagated by cell culture to 10^3 - 10^4

175 inclusion forming units (IFU)/ml and were confirmed as *Chlamydiaceae* by *23S rRNA* gene-based PCR.

176 Sequence analysis of the *16S* and *23S rRNA* signature sequences described by Everett *et al.* (1999a)

177 revealed that all 10 bandicoot isolates belonged to the *C. pneumoniae* species and were more than 99.1%

178 similar to *C. pneumoniae* isolates of human and animal origin, with only 1-5 bp variations.

179

180 **OmpA gene based PCR and sequencing.** All ten bandicoot isolates were tested positive by *C.*

181 *pneumoniae* specific *ompA*-based PCR as per originally described protocol (Apfalter *et al.*, 2003) that

182 confirmed them as *C. pneumoniae*. However, fluorescence signal was approximately 1.7 times lower than

183 in human *C. pneumoniae* control as shown in Figure 1. Increase in annealing temperature resulted in

184 negative fluorescence signal for all bandicoot isolates but did not affect the signal for human *C.*

185 *pneumoniae* control (data not shown). Possible sequence variation between bandicoot and human isolates

186 in the area where published primers and probe meant to bind the target sequence was suspected.

187

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

197

198 **16S rRNA gene sequencing.** *16S rRNA* gene, which is also frequently used as a target for identification
199 and speciation of Chlamydiales and other bacteria, was also amplified and sequenced for all bandicoot
200 isolates. All bandicoot isolates were identical to each other and very similar to human and animal *C.*
201 *pneumoniae* isolates as shown in Table 3. Minor nucleotide differences in both *16S rRNA* and *ompA*
202 genes may indicate genomic diversity within animal *C. pneumoniae* strains, as previously suggested
203 (Hotzel *et al.*, 2001).

204

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

213

214 **Genotyping.** Data on genotyping of human and animal strains of *C. pneumoniae* are limited.

215 For genotyping purposes three genomic targets were selected: variable domain 4 region (VD4) of the
216 *ompA* gene, *YgeD-urk* intergenic spacer and a region of the *rpoB* gene matching *PstI* fragment.

217
218 **VD4 genotyping.** VD4 was proposed as a possible genotyping target due to its small size and high degree
219 of variability between human and animal isolates (Cochrane *et al.*, 2005; Wardrop *et al.*, 1999). Bodetti *et*
220 *al.* (2002) suggested a genotyping system based on as little as 1 bp sequence polymorphism in VD4
221 segment of *ompA* gene. We analyzed *C. pneumoniae* VD4 sequences publicly available in the GenBank
222 database and identified 22 sequences that we separated into 3 human and 7 animal genotype groups
223 (based on their sequence identity within a group) as presented in Table 4. Sequence alignment of the 174
224 bp region of the VD4 segment demonstrated that bandicoot isolates differed from human isolates by 6-7
225 SNPs and were identical to previously reported koala and frog isolates (genotype group A5) and,
226 according to the Cochrane *et al.* (2005) designations, would be assigned to genotype D. Interestingly, the
227 animal *C. pneumoniae* isolates from genotype group A1 from reptiles and amphibians (Bodetti *et al.*,
228 2002) were absolutely identical to human isolates from group H1 as shown in Figure 3.

229
230 ***YgeD-urk* genotyping.** The intergenic spacer region between *YgeD* and *urk* genes differs by the
231 orientation of a 23 bp segment in one of the human *C. pneumoniae* strains (Read *et al.*, 2000) and was
232 used for genotyping of *C. pneumoniae*, (Cochrane *et al.*, 2005). The results of the 320 bp *YgeD-urk* spacer
233 genotyping are shown in Figure 4. The bandicoot isolates were identical to CpnIII genotype recently
234 found in humans (Cochrane *et al.*, 2005) and had the 23 bp invertible region in the same orientation as
235 human TW-183, AR39 and J138 strains. There was a 3 bp difference as compared to human isolates.

236
237 ***RpoB* genotyping.** *RpoB* gene was of relevant interest because of its current use as a target in *C.*
238 *pneumoniae* specific PCR assays (Dowell *et al.*, 2001; Loens *et al.*, 2006). Sequence analysis of the *rpoB*
239 gene region that matched the *PstI* fragment (Campbell *et al.*, 1992) showed a 1 bp polymorphism as
240 compared to human strains of *C. pneumoniae* (Figure 5). This nucleotide change at position 2946 was a
241 silent mutation with no amino acid change (alanine).

242
243 ***C. pneumoniae* species-specific PCR assays and sequence polymorphism.** The results of this study
244 may have important implications for molecular diagnostics of *C. pneumoniae* infection in both animals
245 and humans. Since most of the *C. pneumoniae* specific PCR assays were developed and evaluated using
246 human isolates, they may not be appropriate for detection of animal strains. Even minor sequence
247 variability in the target regions can significantly compromise the sensitivity of the PCR assay and result
248 in false negative results due either to target amplification failure (primer sequence polymorphism) or
249 target detection failure (probe sequence polymorphism) (Kwok *et al.*, 1990; Stevenson *et al.*, 2005). As
250 we demonstrated in this study a real-time *ompA* based PCR assay developed for human strains either
251 generated a lower fluorescent signal or failed to detect bandicoot *C. pneumoniae* isolates because there
252 was one SNP in the forward primer and two SNPs in the probe. Similar assay performance problems due
253 to target sequence variability have been recently reported for real-time PCRs for detection of herpes
254 simplex virus and *Listeria monocytogenes* (Rodriguez-Lazaro *et al.*, 2004; Stevenson *et al.*, 2005). We
255 analyzed several published and currently used *C. pneumoniae* species-specific PCR assays for the
256 presence of the sequence polymorphism in the target regions as shown in Table 5. We found that 6 out of
257 15 species-specific PCR assays had sequence variability in primer and/or probe that may affect their
258 performance in detecting bandicoot strain of *C. pneumoniae*, or possibly other animal and human strains
259 where minor sequence polymorphism may be present.

260
261 The expanding number of animal hosts and isolates of *C. pneumoniae* and genetic similarity of animal
262 and human strains raises some interesting questions about the evolution and epidemiology of this
263 pathogen. It is quite possible that *C. pneumoniae* was primarily an animal pathogen, which was only
264 recently, in evolutionary terms, acquired by humans. Considering almost universal exposure of humans to
265 *C. pneumoniae* and generally inefficient mode of transmission from person to person (Blasi *et al.*, 1998),
266 one could hypothesize that an environmental source of *C. pneumoniae* should be in close proximity to
267 humans. While possible zoonotic transmission of *C. pneumoniae* from exotic animals occupying a unique
268 environmental niche (koalas and bandicoots) to humans seems unlikely, acquisition by contact with

269 animals living in urban or rural areas, including domestic and production animals, either directly or via a
270 transmission vector such as free-living protozoa could be possible (Essig *et al.*, 1997). However, in
271 addition to the previously described equine biovar (Storey *et al.*, 1993) there have been only two reports
272 of possible *C. pneumoniae* infection in domesticated animals. Sako *et al.* (2002) described detection of
273 *C. pneumoniae* antigens in vascular specimens from dogs and Canderle *et al.* (2005) reported high
274 prevalence of *C. pneumoniae* species-specific antibodies in boars.

275
276 More targeted research efforts are necessary to determine the prevalence and host range of *C. pneumoniae*
277 in environmental reservoirs of different geographic regions since most of the published reports on animal
278 strains were simply serendipitous findings. There is also a need for development of relevant and
279 consistent genotyping system to be used for epidemiological surveillance, pathogenesis, evolution studies
280 and characterization of novel *C. pneumoniae* isolates.

281

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284

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420 Table 1. Oligonucleotides used in this study.

421

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

422

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

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

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

430

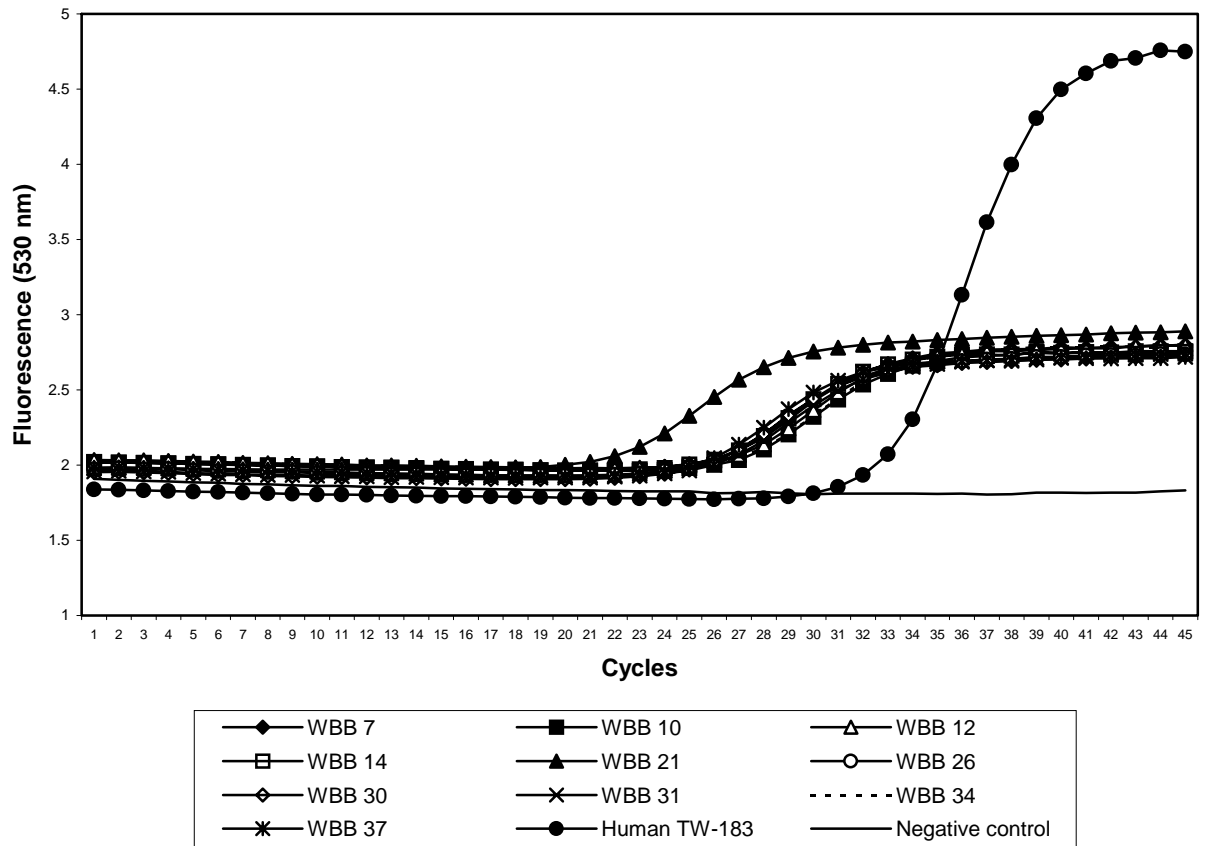
<i>Chlamydophila pneumoniae</i> biovars	<i>ompA</i> gene (1170 bp)	<i>16S rRNA</i> gene (1552 bp)	<i>23S rRNA</i> segment (551 bp)	<i>rpoB</i> segment (687 bp)	<i>ygeD-urk</i> intergenic spacer (320 bp)
Human biovar					
TW-183,	98.2	99.6	99.6	99.9	99.1
AR39	98.2	99.6	99.6	99.9	99.1
J138	98.2	99.4	99.6	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 <i>Chlamydophila</i> species	≤74.6	≤96.3	≤94.7	≤84.0	-§

431

432 * - not available

433

§ – no significant similarity found by BLASTn search



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435
436

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

439
 440 272 356
 441A.....G.....T..... WBB
 442 **GATCCGCTGCTGCAA**ACTATACTACTGCCGTAGATAGACCTAACCCGGCCTACAATAAGCATT**TACACGATGCAGAGTGGTTCAC** Human
 443 **Primer QMOMP1** Probe QMOMPS **Primer QMOMP2**
 444
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 448

449 Fig. 2. Nucleotide sequence alignment of the 85 bp *ompA* target region, used in *C. pneumoniae* specific TaqMan PCR assay. Dots indicate
 450 the same nucleotides as in sequences of human isolates (TW-183, CWL029, AR-39 and J138).

451 Table 4. *C. pneumoniae* genotype groups based on the bp difference at the VD4 locus of the *ompA* gene.
 452

Origin	Genotype group	<i>C. pneumoniae</i> 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

453

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453      826                                                    912
454      ..T.....WBB*
455      TACAGACTAAACTCTTTAGTGCCATACATTGGAGTACAATGGTCTCGAGCAACTTTTGATGCTGATAACATCCGCATTGCTCAGCCA Group H1
456      ..... Group H2
457      ..... Group H3
458      ..... Group A1
459      ..... Group A2
460      ..... Group A3
461      ..G..... Group A4
462      ..T..... Group A5
463      ..T..... Group A6
464      ..T...C.....C..... Group A7
465
466      913                                                    999
467      .....A.G...A...C.....G.....WBB
468      AAACTACCTACAGCTGTTTTAAACTTAACTGCATGGAACCCTTCTTTACTAGGAAATGCCACAGCATTGTCTACTACTGATTCGTTTC Group H1
469      .....C..... Group H2
470      .....G..... Group H3
471      ..... Group A1
472      .....T..... Group A2
473      .....A...C...A..... Group A3
474      .....A...A...A..... Group A4
475      .....A.G...A...C...G..... Group A5
476      .....A.G...A...G...G..... Group A6
477      ..G.....A.....G.G.....TG.T...T.AT....CAA... Group A7
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480 Fig. 3. Nucleotide sequence alignment of a 174 bp region within VD4 segment of bandicoot *C. pneumoniae ompA* gene with human and
481 animal genotype. Dots indicate the same nucleotide as in sequences of human H1 group. GenBank accession number for WBB isolates is
482 DQ358972.

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483
484 1 90
485 .....A.....G..... WBB
486 GGAGTCTTGTACTCCTTTAGGAAACATCTAAGATACCGTAAATCGTTCTTATAAGGCATTCTGACAGTGTTTTTGCTTTTTTAAATCTAT AR39, TW-183, J138
487 .....A.....G..... CWL029
488 .....A.....G..... CpnIII
489
490 91 180
491 .....G..... WBB
492 CTATACGTGAATTGGGGTTTTGTATAACATCCCTTGTATTGGGGGATGAAATATTTTTTTGGATAGAAATCTATCCTAAACTTCGTGA AR39, TW-183, J138
493 .....G..... CWL029
494 .....G..... CpnIII
495
496 181 269
497 .....-..... WBB
498 TCAGAAAATGACTATATCTAAAGATT-TCAATTTCTGATCACGAAGCTTTTTCTTTTGTCTCCTTTTAAATCAATAAAATCTCAAAGGAC AR39, TW-183, J138
499 .....T...A-.C.T..G.T..AG...T..... CWL029
500 .....-..... CpnIII
501
502 270 320
503 ..... WBB
504 CCGCATTTTGTGTGTGCTGATTACAAGAGAAGTACTAAGGAGAAAAATTT AR39, TW-183, J138
505 ..... CWL029
506 ..... CpnIII
507

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508 Fig. 4. Nucleotide sequence alignment of a 320 bp *YgeD-urk* intergenic spacer of bandicoot and human isolates. Dots indicate the same
509 nucleotide as in sequences of human AR39, TW-183 and J138 isolates and dashes represent gaps in the sequences. CpnIII is a
510 *C. pneumoniae* genotype identified in human carotid specimens, GenBank accession number AY427827 (11)

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521
522 2783 2862
523 ..... WBB
524 CCATTATTCACCGTCGTACAGCAGAAAATCGTTGTTTCATGAAGGCCCTACTCTTTGATCAAGAGACAATAGAACGGATAGAA Human*
525 ..... PstI
526 HL-1
527 2863 2942
528 ..... WBB
529 CAAGAAGATTTAGTGGATCTTTTAATGCCTAACTGTGAAATGTATGAAGTGTGAAAGGACTTCTATCAGATTACGAAAC Human
530 ..... PstI
531
532 2943 3022
533 ...C ..... WBB
534 GGCATTACAACGGCTAGAAAATCAATTATAAGACTGAAGTTGAGCATATTCGTGAGGGAGATGCAGATTTAGATCATGGTG Human
535 ..... PstI
536
537 3023 3101
538 ..... WBB
539 TCATTGCGCCAAG-TTAAAGTCTACGTTGCCTCTAAGAGAAAAC TTCAAGTTGGAGATAAAAATGGCTGGACGACACGGAAA Human
540 .....G ..... PstI
541
542 3102 3180
543 ..... WBB
544 TAAAGGTGTTGTTTCCAAAATCGTTCCCGAAGCGGATATGCCATATCTCTAACGGAGAACTGTACAAATGATCC-TG Human
545 .....C.. PstI
546
547 3181 3253
548 ..... WBB
549 AACCCCTCGGGGTGCCTTCAAGGATGAACCTTGGACAGGTATTAGAAACACACC-TAGGTTATGCAGCAAAAA Human
550 .....C.TC .....G .....C PstI
551 HR-1
552

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553 Fig. 5. Nucleotide sequence alignment of a 474 bp region of *rpoB* gene (matching *PstI* fragment) of human and WBB isolates. Dots
554 indicate the same nucleotides as in human isolates and dashes represent gaps in the sequence. Human isolates represented by TW-183,
555 CWL029, AR39 and J138. Primer sequences HL-1 and HR-1 used *C. pneumoniae* specific PCR assay (8) are shown in bold/underlined.

556
557

558 Table 5. Sequence variability in the target regions of the selected species-specific PCR assays as compared to
 559 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	<i>ompA</i> , 185	1	0	2	3	Apfalter <i>et al.</i> , 2003
Real-time	<i>ompA</i> , 134	0	0	0	1	Meijer <i>et al.</i> , 1998
Real-time	<i>ompA</i> , 79	0	0	0	0	Kuoppa <i>et al.</i> , 2002
Real-time	<i>ompA</i> , 109	0	0	2	5	Tondella <i>et al.</i> , 2002
Real-time	<i>ompA</i> , 126	0	0	0	5	Tondella <i>et al.</i> , 2002
Real-time	<i>ompA</i> , 72	0	0	0	0	Kohlhepp <i>et al.</i> , 2005
Nested*	<i>ompA</i> , 333	0/0	0/1	-	4	Tong & Sillis, 1993
Nested	<i>ompA</i> , 497	1/1	0/1	-	13	Lindholt <i>et al.</i> , 1998
Single-step*	<i>rpoB</i> , 438	0	1	-	3	Campbell <i>et al.</i> , 1992
Real-time	<i>rpoB</i> , 82	0	0	0	0	Welti <i>et al.</i> , 2003
Real-time	<i>16S rRNA</i> , 149	0	0	0	2	Meijer <i>et al.</i> , 1998
Single-step*	<i>16S rRNA</i> , 195	0	0	-	0	Madico <i>et al.</i> , 2000
Real-time	<i>16S rRNA</i> , 195	0	0	0	2	Hardick <i>et al.</i> , 2004
Single-step*	<i>16S rRNA</i> , 464	0	0	-	2	Gaydos <i>et al.</i> , 1992
Real-time	<i>16S rRNA</i> , 154	1	0	0	2	Templeton <i>et al.</i> , 2005

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