Isolation and Antimicrobial Susceptibilities of Chlamydial Isolates from Western Barred Bandicoots

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A range of species of Chlamydiales has previously been detected in a variety of Australian marsupials, including koalas and western barred bandicoots. Thirty-seven ocular, urogenital, or nasal swabs were obtained from 21 wild barred bandicoots. Chlamydia culture and antibiotic susceptibility testing were performed for cycloheximide-treated HEp-2 cells in 96-well microtiter plates. Chlamydia spp. were isolated from 11 specimens from 9 (42.3%) bandicoots. All isolates were identified as Chlamydiales by conventional PCR with 16S and 23S rRNA gene primers specific to Chlamydiales and were confirmed to be Chlamydia pneumoniae by a C. pneumoniae-specific ompA-based real-time PCR assay and 16S rRNA and 23S rRNA gene signature sequence analyses. The MICs of azithromycin, doxycycline, ciprofloxacin, and enrofloxacin for 10 C. pneumoniae isolates from these bandicoots ranged from 0.015 to 1 µg/ml, 0.25 to 1 µg/ml, 0.25 to 2 µg/ml, and 0.25 to 0.5 µg/ml, respectively. The MICs at which 90% of isolates were inhibited and the minimal bactericidal concentrations were within the ranges reported previously for human isolates of C. pneumoniae.
for 5 min at 72°C. PCR products were purified with a QIAGEN QIAquick PCR purification kit and were sequenced in both directions (GeneWiz, North Brunswick, NJ).

(ii) C. pneumoniae-specific real-time PCR TaqMan assay. Chlamydial isolates were further tested using a C. pneumoniae-specific ompA-based real time PCR as a second and concurrent test to detect the presence of C. pneumoniae. The C. pneumoniae-specific primers and probe targeting an 85-bp highly conserved and specific region of the ompA gene were as follows: primers QMOMP1 (5′-GATCGGCTGC TGCAAACGTACT-3′) and QMOMP2 (5′-GTGAAACCACCTGCATGTAA-3′) and probe QMOMPS (5′-6-carboxyfluorescein–TAGGCCGGTCTATCTACGGCAGT–6-carboxytetramethylrhodamine–3′) (1). Real-time PCR was performed using a Roche LightCycler (version 2.0) at 95°C for 10 min, followed by 45 cycles of 95°C for 5 s, 65°C or 60°C for 10 s, and 72°C for 10 s. A low-titer TW-183 isolate of C. pneumoniae (ATCC VR-2282) was used as a positive control, and double-distilled water was used as a negative control, in all the in vitro experiments, PCRs, and sequencing reactions.

Sequence analysis. The sequences were analyzed using BLAST 2 (http://www.ncbi.nlm.nih.gov/BLAST/b2seq/) and were compared to the sequences of 16S and 23S rRNA genes of Chlamydiales available in GenBank, including the three previously described biovars of C. pneumoniae.

Nucleotide sequence accession numbers. The signature sequences of the 16S rRNA and 23S rRNA genes from bandicoot C. pneumoniae are currently available in GenBank under accession numbers DQ444323 and DQ465990, respectively.

RESULTS

Culture. Thirty-seven ocular, throat, and cloacal specimens were obtained from 21 bandicoots. A total of 11 specimens from 9 (42.8%) bandicoots were positive for Chlamydia spp. after 3 to 6 passages, confirming the presence of viable chlamydiae in the specimens. Inclusions varied in size and granularity but were very similar in appearance to those of human isolates (Fig. 1). Of the 11 positive swabs, 4 were obtained from the left eye, 3 from the right eye, 1 from the nose, and 3 from the throat.

16S and 23S rRNA gene-based PCR and sequence analysis. PCR was performed on 10 of the 11 culture-positive specimens. All 10 specimens were shown to belong to the Chlamydiales by 16S and 23S rRNA gene primers specific to Chlamydiales. Sequence analysis of 16S and 23S rRNA gene signature regions revealed that all 10 bandicoot isolates were identical to each other. BLAST analysis of the 551-bp segment of the 23S rRNA gene signature sequence demonstrated that bandicoot isolates were 99.6% and 99.1% identical to human (2-bp difference) and equine (5-bp difference) biovars of C. pneumoniae, respectively. BLAST analysis of the 294-bp 16S rRNA gene signature segment showed that bandicoot isolates were 99.3% identical to the human biovar (2-bp difference), 99.5% identical to the koala biovar (1-bp difference), and 98.9% identical to the equine biovar (3-bp difference) (GenBank accession numbers AE 017160, AF 100957, and U68426, respectively).

ompA-based real-time PCR. All 10 isolates tested positive by a C. pneumoniae-specific ompA-based PCR assay, confirming the identity of the isolates as C. pneumoniae.

In vitro susceptibility testing. The MIC50, MIC90, and MBC90 values for bandicoot isolates of C. pneumoniae are shown in Table 1. The ranges of MICs and MBCs were 0.015 to 1 μg/ml for azithromycin, 0.25 to 1 μg/ml for doxycycline, 0.25 to 2 μg/ml for ciprofloxacin, and 0.25 to 0.5 μg/ml for enrofloxacin.
TABLE 1. In vitro antibiotic susceptibilities of 11 bandicoot isolates of C. pneumoniae

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (mg/liter)</th>
<th>MBC (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range 50% 90%</td>
<td>Range 90%</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0.015–1</td>
<td>0.015–1</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.25–1</td>
<td>0.25–1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.25–2</td>
<td>0.25–2</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>0.25–0.5</td>
<td>0.25–0.5</td>
</tr>
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</table>

**DISCUSSION**

We were able to isolate *Chlamydia* spp. in tissue culture from multiple anatomic sites from 42.8% of bandicoots in this sample. Tissue culture using cycloheximide-treated HEp-2 cells appears to be an effective system for isolation and propagation of these organisms. The isolates were propagated for further analysis by PCR. All isolates were confirmed to be *Chlamydiales* by staining in cell culture with monoclonal antibodies specific to the *Chlamydiaceae* and by conventional PCR with 16S and 23S rRNA gene primers specific to *Chlamydiales*, and they were subsequently identified as *C. pneumoniae* by a highly species specific *ompA*-based real-time PCR assay using specific primers and a specific probe. In addition, 16S rRNA and 23S rRNA signature sequence analysis revealed similarities of 99.3% and 99.6%, respectively, to human isolates of *C. pneumoniae*; similarity of more than 95% is enough to confirm the species (7). *C. pneumoniae* is considered to be a primarily human respiratory pathogen, although recent reports have identified koalas, frogs (2, 11, 12), and horses (15) as additional hosts and have detected the koala biobur in human carotid plaque specimens obtained from patients undergoing elective endarterectomy in Australia (6). The identification of *C. pneumoniae* infections in bandicoots provides further evidence for the expanding host range of this species. The identification of novel hosts and biobars of *C. pneumoniae* raises interesting questions about the evolution and epidemiology of this pathogen, specifically the presence of animal reservoirs and additional modes and or directions of transmission across species. Additional studies are needed to gain further understanding of these issues.

Although molecular techniques have been increasingly used in the past few years for the detection of sequences of *Chlamydiaceae* in a wide range of animal and environmental sources, and although these techniques, in combination with sequencing, have facilitated the discovery of novel *Chlamydiaceae* (3) and an increasing range of chlamydial hosts, isolation and propagation of the organism in culture allows further antigenic and molecular characterization of isolates and remains essential for determination of in vitro susceptibilities. Several of the animals in this study were initially treated with beta-lactam antibiotics without response but subsequently responded to intramuscular tetracycline. In vitro testing of the susceptibilities of these *C. pneumoniae* isolates to azithromycin, ciprofloxacin, and doxycycline demonstrated antibiotic susceptibilities similar to those reported for human isolates (9).

Enrofloxacin, a veterinary quinolone, has been demonstrated to be very active against *Chlamydia psittaci* in vitro (5) and has been used for the treatment and prevention of psittacosis in birds (13). Enrofloxacin has not been tested against *C. pneumoniae*. Although in vitro susceptibilities may not necessarily predict in vivo efficacy, these data indicate a possible role for enrofloxacin in the treatment of *C. pneumoniae* infections in Australian marsupials.

**ACKNOWLEDGMENTS**

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**REFERENCES**