Isolation and Antimicrobial Susceptibilities of Chlamydial Isolates from Western Barred Bandicoots

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Title: Isolation and Antimicrobial Susceptibilities of Chlamydial Isolates from Western Barred Bandicoots

Running title: Chlamydial Infections in Western Barred Bandicoots

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Abstract

A range of *Chlamydiales* species have previously been detected in a variety of Australian marsupials, including koalas and western barred bandicoots. Thirty-nine ocular, urogenital or nasal swabs were obtained from 37 wild western barred bandicoots. Chlamydia culture and antibiotic susceptibility testing were performed in cycloheximide treated HEp-2 cells in 96 well microtiter plates. Chlamydia spp were isolated from 11 specimens from 9 (24.3%) bandicoots. All isolates were identified as *Chlamydiales* by *Chlamydiales* specific *16S* and *23S rRNA* primers using conventional polymerase chain reaction (PCR) and confirmed to be *Chlamydia pneumoniae* by a *C. pneumoniae* specific *ompA* based real-time PCR assay and *16S rRNA* and *23S rRNA* signature sequence analyses. The MICs of azithromycin, doxycycline, ciprofloxacin and enrofloxacin of 10 *C. pneumoniae* isolates from these bandicoots ranged from 0.015-1 μg/ml, 0.25-1 μg/ml, 0.25-2 μg/ml and 0.25-0.5 μg/ml respectively. The MIC_{90} and minimal bactericidal concentrations (MBC) were within the range reported previously for human isolates of *C. pneumoniae.*

Introduction

The range of hosts infected by *Chlamydiales* has been expanding. Recent studies using a combination of molecular methods and cell culture have identified several existing and some novel *Chlamydiales* species infecting a wide range of Australian marsupials including koalas and western barred bandicoots (3). Formerly found over much of Western Australia, western barred bandicoots (*Perameles bougainville*) are now an endangered species found in the wild only on Bernier and Dorre Islands off the coast of Western Australia (8). Detection of chlamydial organisms including *Chlamydia pecorum* and unidentified *Chlamydiales* in these animals has previously been associated with clinical diseases in the form of conjunctivitis, lid proliferation, corneal opacity as well as an asymptomatic state (16). The in vitro susceptibilities of Chlamydia spp isolated from Australian marsupials, including bandicoots, have not been reported. The purpose of this study was to isolate chlamydia species in tissue culture from specimens obtained from these unique hosts and to determine the growth characteristics, species and in vitro antibiotic susceptibilities.

Methods

Specimen collection. Ocular, urogenital or nasal swabs were obtained from western barred bandicoots from Dryandra, Western Australia (WA) and

Bernier Islands, WA. All animals sampled were from wild populations. Thirteen of these animals showed clinical signs of disease in the form of conjunctivitis, proliferation of the eyelid and possible pneumonia. Five were noted to have cataracts or corneal scarring suggestive of previous ocular disease.

Cell culture. Isolation of chlamydia spp. was performed in cycloheximide-treated HEp-2 cells grown in 96-well microtiter plates as previously described (14). Culture confirmation was done by staining with a chlamydia genus specific fluorescein-conjugated monoclonal antibody (Pathfinder Chlamydia Confirmation System, Kallestad Diagnostics, Chaska, MN). Each specimen was passaged up to 6 times. Isolates that were positive by culture were propagated for further characterization including susceptibility testing and molecular diagnostic studies.

In vitro susceptibility testing. Susceptibility testing was performed in cell culture using HEp-2 cells as previously described (10). Azithromycin (Pfizer Central Research, Groton, CT), ciprofloxacin (Miles Pharmaceuticals, West Haven, CT), enrofloxacin (Bayer Corp., Shawnee Mission, KS) and doxycycline were supplied as powders and solubilized according to the manufacturers' instructions. Each well was inoculated with 0.1 ml of the test isolate diluted to yield 10^3 to 10^4 inclusion-forming units per ml, centrifuged at 900 × *g* for 1 hour, and then incubated at 35°C for 1 hour. Wells were then

aspirated and overlaid with 0.2 ml of overlay medium containing 1 μg/mL of cycloheximide and serial twofold dilutions of the test drug. After incubation at 35°C for 72 hours, cultures were fixed and stained for inclusions with fluorescein-conjugated antibody to the lipopolysaccharide genus antigen. The minimum inhibitory concentration (MIC) was the lowest antibiotic concentration at which no inclusions were seen. The minimal bactericidal concentration (MBC) was determined by aspirating the antibiotic-containing medium, washing wells twice with phosphate-buffered saline and adding antibiotic-free medium. Cultures were frozen at −70°C, thawed, passed onto new cells and incubated for 72 hours and then fixed and stained as described above. The MBC was considered to be the lowest antibiotic concentration, which resulted in no inclusions after passage. All tests were run in triplicate.

DNA extraction. DNA from culture positive isolates was extracted using the DNeasy tissue kit (Qiagen, Valencia, CA), as per manufacturer's instructions and stored at -20°C prior to PCR analysis.

***C. pneumoniae* species identification**

***1. 16S* and *23S rRNA* genes signature sequencing.** Amplification/sequencing primers used for *16S rRNA* gene and *23S rRNA* gene products were 16SIGF 5'-CGGCGTGGATGAGGCAT-3', 16SIGR 5'- TCAGTCCCAGTGTTGGC-3', U23F 5'-GATGCCTTGGCATTGATAGGC GATGAAGGA-3' and 23SIGR 5'-TGGCTCATCATGCAAAAGGCA-3', as described by Everett et

al (7). PCR was performed using Qiagen ProofStart DNA polymerase kit, at 95°C x 5 min, then 45 cycles of 94°C x 30 sec, 60°C x 30 sec, 72°C x 60 sec, with a final extension for 5 min at 72°C. PCR products were purified with Qiagen QIAquick PCR Purification kit and sequenced in both directions (GeneWiz, North Brunswick, NJ).

2. ***C. pneumoniae*** **specific real-time PCR TaqMan assay**. The *C. pneumoniae* specific primers and probe targeting a 85 bp highly conserved and specific region of the *ompA* gene were as follows: QMOMP1 5'-GATCCGC TGCTGCAAACTATACT-3', QMOMP2 5'-GTGAACCACTCTGCATCGTGTAA-3', probe QMOMPS 5'- FAM-TAGGCCGGGTTAGGTCTATCTACGGCAGT-TAMRA-3' (1). Real-time PCR was performed using the Roche LightCycler version 2.0, at 95°C x 10 min, then 45 cycles of 95°C x 5 sec, 65°C or 60°C x 10 sec, 72°C x 10 sec. 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/bl2seq/ bl2.html] and compared to the *Chlamydiales* sequences of *16S rRNA* and *23S rRNA* available in GenBank, including three biovars of *C. pneumoniae*.

Results

Culture. Thirty-nine ocular, throat, and cloacal specimens were obtained from 37 bandicoots. A total of 11 (28.2%) specimens from 9 (24.3%) bandicoots were positive for chlamydia spp after 3-6 passages. Inclusions varied in size and granularity, but were very similar in appearance to those of human isolates. Of the 11 positive swabs, 4 were obtained from the left eye, 2 from the right eye, 1 from the nose and 3 from the throat. Source site of one swab was not clear from the label.

***16S* and *23S rRNA* gene based PCR.** PCR was performed on 10 of the 11 culture positive specimens. All 10 specimens were shown to belong to *Chlamydiales* by *Chlamydiales* specific *16S* and *23S rRNA* primers.

***OmpA* based real time PCR**. All 10 isolates tested positive by *C. pneumoniae* specific *ompA* based PCR assay.

Sequence analysis. Sequence analysis of *16S* and *23S rRNA* genes signature regions revealed that all 10 bandicoot isolates were identical to each other. The signature sequences of the *16S rRNA and 23S rRNA* genes from bandicoot *C. pneumoniae* are currently available in GenBank, accession numbers DQ444323 and DQ465990, respectively. As shown in Table 2, BLAST analysis of the 551 bp segment of *23S rRNA* gene signature sequence demonstrated that bandicoot isolates were 99.6 % and 99.1 % identical to human (2 bp difference) and equine biovar (5 bp) of *C. pneumoniae*,

respectively. BLAST analysis of the 294 bp *16S rRNA* gene signature segment showed that bandicoot isolates were 99.3% identical to human biovar (2 bp variation), 99.5% to koala biovar (1 bp) and 98.9% to equine biovar (3 bp).

In vitro susceptibility testing. The MIC_{50}, MIC_{90} and MBC_{90} values for bandicoot isolates of *C. pneumoniae* are shown in Table 1. The range of MICs and MBCs for azithromycin was 0.015-1 µg/ml, doxycycline 0.25-1 µg/ml, ciprofloxacin 0.25-2 µg/ml and enrofloxacin 0.25-0.5 µg/ml.

Discussion

We were able to isolate chlamydia spp in tissue culture from multiple anatomic sites from 24% 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 with *Chlamydiaceae* specific monoclonal antibodies in cell culture, *Chlamydiales* specific *16S rRNA* and *23S rRNA* PCR and 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 a similarity of 99.3% and 99.6 % respectively to human isolates of *C. pneumoniae*; similarity 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. Furthermore, the koala biovar, which has previously been isolated from koalas and frogs, was recently identified in human carotid plaque specimens obtained from patients undergoing elective endarterectomy for carotid artery disease in Australia (6). The identification of *C. pneumoniae* infections in bandicoots provides further evidence for the expanding diversity of this species. The identification of an increasing number of hosts and novel biovars of *C. pneumoniae* raises some 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 detection of *Chlamydiales* sequences in a wide range of animal and environmental sources, and in combination with sequencing have facilitated discovery of novel *Chlamydiales* (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 susceptibility

testing 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 in vitro against *C. psittaci* (5) and has been used for 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 this drug in the treatment of *C. pneumoniae* infections in Australian marsupials.

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Table 1. In vitro antibiotic susceptibilities of 11 bandicoot isolates of *C. pneumoniae*

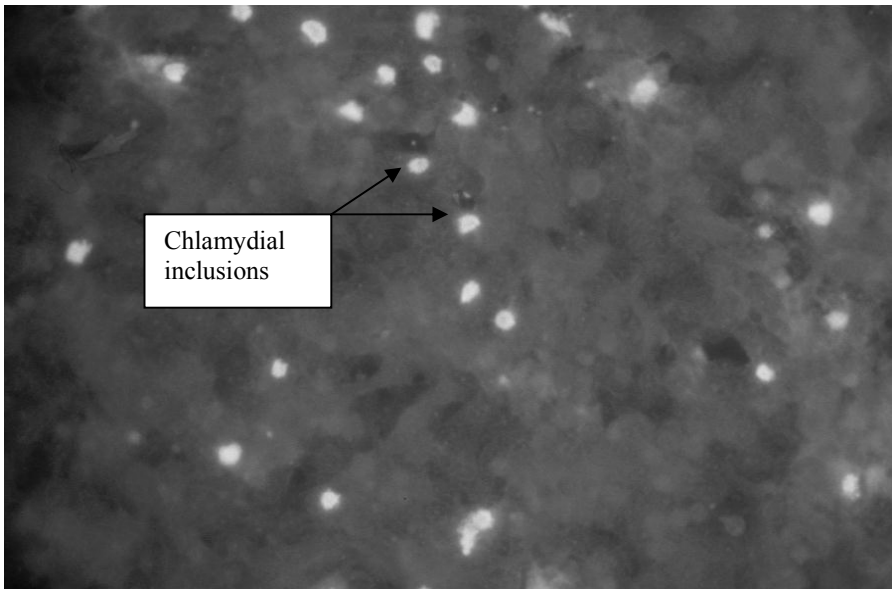
Drug	MIC (mg/l)[+]			MBC (mg/l)[+]	
	Range	50%	90%	Range	90%
Azithromycin	0.015-1	0.5	1	0.015-1	1
Doxycycline	0.25-1	0.25	1	0.25-1	1
Ciprofloxacin	0.25-2	0.5	2	0.25-2	2
Enrofloxacin	0.25-0.5	0.5	0.5	0.25-0.5	0.5

[+]50 and 90% MIC (or MBC) for 50 % and 90% of strains respectively.

Table 2. Signature sequence similarities of Western Barred Bandicoot *C. pneumoniae* isolates to existing *C. pneumoniae* biovars

Chlamydia pneumoniae	*16S rRNA* (294 bp)	*23S rRNA* (551 bp)
Human biovar	99.3 (2 bp)*	99.6(2 bp)
Koala biovar	99.5 (1 bp)	Not available
Equine biovar	98.9 (3 bp)	99.1 (5 bp)
Other *Chlamydia* species	≤94.5% (≥16 bp)	≤94.7 (≥29 bp)

* % Similarity (single nucleotide polymorphisms)



Photomicrograph of a bandicoot *C. pneumoniae* isolate showing characteristic chlamydial inclusions in Hep-2 cell culture under 200x magnification