

## Isolation and Antimicrobial Susceptibilities of Chlamydial Isolates from Western Barred Bandicoots<sup>∇</sup>

Swati Kumar,<sup>1\*†</sup> Andrei Kutlin,<sup>1†</sup> Patricia Roblin,<sup>1</sup> Stephan Kohlhoff,<sup>1</sup> Tracey Bodetti,<sup>2</sup> Peter Timms,<sup>2</sup> and Margaret R. Hammerschlag<sup>1</sup>

Department of Pediatrics, Division of Infectious Diseases, State University of New York, Downstate Medical Center, 450 Clarkson Ave., Brooklyn, New York 11203,<sup>1</sup> and School of Life Sciences, Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Queensland 4000, Australia<sup>2</sup>

Received 21 August 2006/Returned for modification 10 October 2006/Accepted 7 November 2006

**A range of species of *Chlamydiales* have 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 western 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 (42.8%) 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*.**

The range of hosts known to be infected by *Chlamydiales* has been expanding. Recent studies using a combination of molecular methods and cell culture have identified several existing and some novel species of *Chlamydiales* infecting a wide range of Australian marsupials, including koalas and western barred bandicoots (3, 4). 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, eyelid proliferation, and 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 their growth characteristics, species, and *in vitro* antibiotic susceptibilities.

### MATERIALS AND METHODS

**Specimen collection.** Ocular, urogenital, or nasal swabs were obtained from western barred bandicoots from Dryandra, Western Australia, and Bernier Islands, Western Australia. All animals sampled were from wild populations. Thirteen of these animals showed clinical signs of disease in the form of con-

junctivitis, proliferation of the eyelid, and possible pneumonia. Five were noted to have cataracts or corneal scarring suggestive of previous ocular disease.

**Cell culture.** *Chlamydia* spp. were isolated 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 found 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, 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<sup>3</sup> to 10<sup>4</sup> inclusion-forming units per ml, centrifuged at 900 × g for 1 h, and then incubated at 35°C for 1 h. 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 h, cultures were fixed and stained for inclusions with a fluorescein-conjugated antibody to the lipopolysaccharide genus antigen. The 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, incubated for 72 h, and then fixed and stained as described above. The MBC was defined as the lowest antibiotic concentration that resulted in no inclusions after passage. The MIC<sub>90</sub> and MBC<sub>90</sub> were defined as the concentrations of the antibiotic that inhibited or killed 90% of the isolates tested, respectively. All tests were run in triplicate.

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

***C. pneumoniae* species identification. (i) 16S and 23S rRNA gene signature sequencing.** The 16S and 23S rRNA gene-based signature sequences were determined in order to identify chlamydial isolates to the species level, as recommended by Everett et al. (7). The amplification/sequencing primers used for 16S rRNA and 23S rRNA gene products were 16SIGF (5'-CGGCGTGGATGAGG CAT-3'), 16SIGR (5'-TCAGTCCCAGTGTGGC-3'), U23F (5'-GATGCCTT GGCATTGATAGGCGATGAAGGA-3'), and 23SISR (5'-TGGCTCATCAT GCAAAAGGCA-3'), as described by Everett et al. (7). PCR was performed, using a QIAGEN ProofStart DNA polymerase kit, at 95°C for 5 min, followed by 45 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s, and a final extension

\* Corresponding author. Mailing address: Department of Pediatrics, Division of Infectious Diseases, State University of New York, Downstate Medical Center, 450 Clarkson Ave., Brooklyn, NY 11203. Phone: (718) 270-7588. Fax: (718) 270-3210. E-mail: swati.kumar@downstate.edu.

† S.K. and A.K. contributed equally to this work.

∇ Published ahead of print on 22 November 2006.

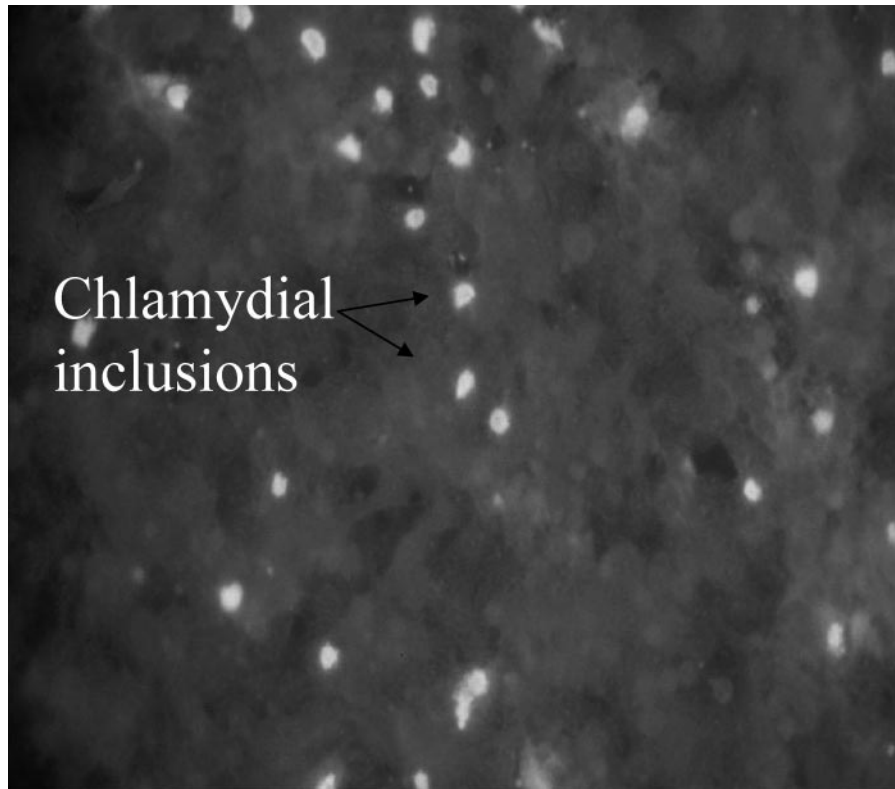


FIG. 1. Photomicrograph of a bandicoot *C. pneumoniae* isolate showing characteristic chlamydial inclusions in HEP-2 cell culture. Magnification,  $\times 200$ .

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'-GATCCGCTGCTGCAAACTATACT-3') and QMOMP2 (5'-GTGAACCACTCTGCATCGTGTAA-3') and probe QMOMPS (5'-6-carboxyfluorescein-TAGGCCGGTGTAGGTCTATCTACGGCAGT-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/bl2seq/>) 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 MIC<sub>50</sub>, MIC<sub>90</sub>, and MBC<sub>90</sub> values for bandicoot isolates of *C. pneumoniae* are shown in Table 1. The ranges of MICs and MBCs were 0.015 to 1  $\mu\text{g/ml}$  for azithromycin, 0.25 to 1  $\mu\text{g/ml}$  for doxycycline, 0.25 to 2  $\mu\text{g/ml}$  for ciprofloxacin, and 0.25 to 0.5  $\mu\text{g/ml}$  for enrofloxacin.

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

Drug	MIC (mg/liter)			MBC (mg/liter)	
	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

## 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 biovar 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 biovars 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 *Chlamydiales* in a wide range of animal and environmental sources, and although these techniques, in combination with sequencing, have facilitated the 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 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

We thank the Australian veterinarians who did the field work and collected the specimens.

## REFERENCES

1. Apfalter, P., W. Barousch, M. Nehr, A. Makristathis, B. Willinger, M. Rotter, and A. M. Hirschl. 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.
2. Berger, L., K. Volp, S. Mathews, R. Speare, and P. Timms. 1999. *Chlamydia pneumoniae* in a free-ranging giant barred frog (*Mixophyes iteratus*) from Australia. *J. Clin. Microbiol.* **37**:2378–2380.
3. Bodetti, T. J., K. Viggers, K. Warren, R. Swan, S. Conaghty, C. Sims, and P. Timms. 2003. Wide range of *Chlamydiales* types detected in native Australian mammals. *Vet. Microbiol.* **96**:177–187.
4. Bodetti, T. J., E. Jacobson, C. Wan, L. Hafner, A. Pospischil, K. Rose, and P. Timms. 2002. Molecular evidence to support the expansion of the host range of *Chlamydia pneumoniae* to include reptiles as well as humans, horses, koalas and amphibians. *Syst. Appl. Microbiol.* **25**:146–152.
5. Butaye, P., R. Ducatelle, P. D. Backer, H. Vermeersch, J. P. Remon, and F. Haesebrouck. 1997. In vitro activities of doxycycline and enrofloxacin against European *Chlamydia psittaci* strains from turkeys. *Antimicrob. Agents Chemother.* **41**:2800–2801.
6. Cochrane, M., P. Walker, H. Gibbs, and P. Timms. 2005. Multiple genotypes of *Chlamydia pneumoniae* identified in human carotid plaque. *Microbiology* **151**:2285–2290.
7. Everett, K. D. E., R. M. Bush, and A. A. Anderson. 1999. 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.
8. Friend, J. A., and A. A. Burbidge. 1995. Western barred bandicoot, *Perameles bougainville*, p. 178–180. In R. Strahan (ed.), *Mammals of Australia*. Reed Books, Sydney, Australia.
9. Hammerschlag, M. R. 1994. Antimicrobial susceptibility and therapy of infections caused by *Chlamydia pneumoniae*. *Antimicrob. Agents Chemother.* **38**:1873–1878.
10. Hammerschlag, M. R., C. L. Hyman, and P. M. Roblin. 1992. In vitro activities of five quinolones against *Chlamydia pneumoniae*. *Antimicrob. Agents Chemother.* **36**:682–683.
11. Hotzel, H., E. Grossman, F. Mutschmann, and K. Sachse. 2001. Genetic characterization of a *Chlamydia pneumoniae* isolate from an African frog and comparison to currently accepted biovars. *Syst. Appl. Microbiol.* **24**:63–66.
12. Jackson, M., N. White, P. Griffard, and P. Timms. 1999. Epizootiology of *Chlamydia* infections in two free-range koala populations. *Vet. Microbiol.* **65**:255–264.
13. Lindenstruth, H., and J. W. Frost. 1993. Enrofloxacin (Baytril)—an alternative for psittacosis prevention and therapy in imported psittacines. *Dtsch. Tierarztl. Wochenschr.* **100**:364–368.
14. Roblin, P. M., W. Dumornay, and M. R. Hammerschlag. 1992. Use of HEp-2 cells for improved isolation and passage of *Chlamydia pneumoniae*. *J. Clin. Microbiol.* **30**:1968–1971.
15. Storey, C., M. Lusher, P. Yates, and S. Richmond. 1993. Evidence for *Chlamydia pneumoniae* of non-human origin. *J. Gen. Microbiol.* **139**:2621–2626.
16. Warren, K., R. Swan, T. Bodetti, T. Friend, S. Hill, and P. Timms. 2005. Ocular *Chlamydiales* infections of western barred bandicoots (*Perameles bougainville*) in Western Australia. *J. Zoo Wildl. Med.* **36**:100–102.