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# PREVALENCE AND PATHOLOGIC FEATURES OF *CHLAMYDIA PECORUM* INFECTIONS IN SOUTH AUSTRALIAN KOALAS (*PHASCOLARCTOS CINEREUS*)

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ABSTRACT: *Chlamydia pecorum* infection is highly prevalent in many koala (*Phascolarctos cinereus*) populations in the eastern states of Australia, causing ocular and urogenital tract disease. In contrast, the current prevalence of chlamydiosis in South Australian (SA) koalas is largely unknown, with few reports of clinical cases. We examined 65 SA rescued wild koalas at necropsy and collected ocular and urogenital swabs for the detection of *C. pecorum* by PCR. We detected *C. pecorum* in ocular or urogenital swabs from 57 koalas (88%), and 34 koalas were positive at both ocular and urogenital sites. Clinically overt chlamydial disease was present in only 12 (21%) positive koalas. Gross lesions were often externally inapparent as they affected the urogenital tract ($n$=5), and 24 infected koalas had microscopically evident lesions only. Lesions were predominantly mild and included conjunctivitis, cystitis, and urethritis. Reproductive tract disease was infrequently observed. We detected *C. pecorum* in 16 (28%) koalas with no evidence of chlamydial disease, suggesting the presence of subclinical carriers in this population. Based on these findings, chlamydiosis has a higher occurrence in SA koala populations than previously thought, but is most often mild and does not always result in overt clinical disease; inapparent and subclinical infections appear common. Further studies of the prevalence in wild-caught SA koalas are needed along with research into the host and bacterial factors that may influence disease outcome in these animals.

*Key words:* Chlamydiosis, histopathology, koala, polymerase chain reaction, postmortem.

## INTRODUCTION

Chlamydiosis, caused by the obligate intracellular bacteria *Chlamydia*, is the most documented disease of koalas (*Phascolarctos cinereus*), with a prevalence of up to 100% in some koala populations in the eastern states of Australia (Polkinghorne et al. 2013). Two species of *Chlamydia* are known to infect koalas: *Chlamydia pecorum* and *Chlamydia pneumoniae*. *Chlamydia pneumoniae* causes pneumonia and rhinitis; however, *C. pecorum* is more common and pathogenic, causing keratoconjunctivitis, cystitis, pyometra, ovarian cysts, and vaginitis, potentially leading to infertility in female koalas (Higgins et al. 2005b; Polkinghorne et al. 2013; Burach et al. 2014). Chlamydiosis in male koalas has been less extensively studied, but findings suggest that infection may also lead to infertility in males (Deif 2011). Although the method of transmission has not been confirmed, it is generally accepted that urogenital infection is sexually transmitted and that mothers infect offspring through close contact (Jackson et al. 1999; Polkinghorne et al. 2013). Ocular infection may be directly transmitted through male-to-male aggression and potentially by arthropod vectors (Jackson et al. 1999; Whittington 2001; Polkinghorne et al. 2013).

*Chlamydia* infection can be split into three categories: subclinical, overt disease, and inapparent overt disease (Polkinghorne et al. 2013). In subclinical infection there are no signs of disease; overt disease denotes the presence of obvious external clinical signs such as conjunctivitis and "wet bottom"; inapparent overt disease describes lesions that are not clinically obvious, such as of the urogenital tract, which can only be detected using ultrasound, postmortem examination, or histopathology (Polkinghorne et al. 2013).

*Chlamydia* in New South Wales and Queensland koala populations is well studied due to the high levels of severe clinical disease (Wan et al. 2011); however, prevalence in South Australian (SA) koalas is unknown, with only a few mild clinical cases of ocular chlamydiosis reported recently (Funnell et al. 2013). Studies based on samples collected in 1998–2000 found high prevalence of chlamydial infection in the Mount Lofty population (Polkinghorne et al. 2013) but, due to the lack of reported clinical cases, current prevalence is thought to be low, confounding efforts to effectively manage these populations. We describe postmortem examination and histopathology findings for koalas tested for *C. pecorum* by PCR and assess the prevalence in rescued wild SA koalas and the occurrence of clinical and subclinical disease.

## MATERIALS AND METHODS

### Animals

We conducted postmortem examination of 65 rescued wild koalas from the Mount Lofty Ranges ($n$=62) and Eyre Peninsula ($n$=3) populations at the School of Animal and Veterinary Sciences, University of Adelaide, during 2012 ($n$=32) and 2013 ($n$=33) (Department of Environment, Water and Natural Resources permit Y26054). These koalas had died or were euthanized for humane reasons due to trauma or illness. Of the 65 koalas, 40 were male and 25 female. Age was estimated by the tooth-wear class (TWC) system (Martin 1981; Martin and Handasyde 1990). There were 15 koalas in TWC I (koalas approximately 1–2 yr old), 22 in TWC II (2–3 yr), 10 in TWC III (4 yr), 12 in TWC IV (5–6 yr), two in TWC V (10–12 yr), and four in TWC VI (12+ yr). Body condition score (BCS) was determined by palpation of the musculature over the scapulae using scores of 1 to 5, representing poor to excellent (Blanshard and Bodley 2008). Seven koalas were in BCS 1, 24 in BCS 2, 13 in BCS 3, 14 in BCS 4, and seven in BCS 5.

### Pathology

Koalas underwent routine postmortem examination and detected abnormalities were recorded. Dry swabs (Copan, Brescia, Italy) were taken of the left and right conjunctiva and the urogenital sinus (female) and penile urethra (male) and frozen at –20 C pending PCR analysis. Ocular and urogenital tract tissues (conjunctiva, bladder, ovary, uterus, vagina [female], and prostate, penis, testis [male]) were fixed in 10% neutral-buffered formalin and processed routinely. We examined H&E-stained tissue sections and recorded histopathologic changes without knowledge of molecular test results.

### *Chlamydia pecorum*–specific PCR screening

Swabs were processed and tested for *C. pecorum* using a species-specific PCR targeting the 16S rDNA described by Mathew et al. (2013), except that reactions were prepared with water instead of SYBR Green reagent. Samples yielding a specific 202–base pair PCR product following agarose gel electrophoresis and visualization under an ultraviolet transilluminator were considered PCR positive. The PCR sensitivity of this conventional assay was confirmed at 20 *C. pecorum* genome copies, following serial dilution of a known concentration of *C. pecorum* MarsBar isolate gDNA, previously quantified by the *C. pecorum*–specific quantitative PCR assay (data not shown; Mathew et al. 2013). Controls included in each PCR run were distilled-water template controls and diluted koala *C. pecorum* MarsBar PCR gDNA as positive controls.

### Statistical analysis

Positive PCR, histopathology, and postmortem examination findings were analyzed by sex, TWC, and BCS using chi-squared tests in Excel (Microsoft, North Ryde, New South Wales, Australia). Where needed, post hoc tests were used to detect differences between groups. Statistical significance was assigned at $P<0.05$.

## RESULTS

### PCR prevalence and epidemiologic factors

Ocular or urogenital swabs were PCR positive for 57 of 65 (88%) rescued wild koalas. Of those koalas that were positive, 50/57 (88%) had positive ocular swabs (left and/or right eye) and 40/57 (70%) had positive urogenital (sinus or penile urethra) swabs. Thirty-four of 57 (60%) affected koalas had positive PCR results for both the ocular and urogenital sites. Of the Mount Lofty population, 55 koalas were positive (89%), whereas 2/3 Eyre Peninsula koalas were PCR positive for both ocular and urogenital swabs.

Thirty-seven of 40 (93%) male koalas tested had PCR-positive swab results, as did 20/25

TABLE 1. Disease status and pathologic findings in South Australian koalas (*Phascolarctos cinereus*) infected with *Chlamydia pecorum* as determined by PCR of ocular and urogenital swabs.

| Disease status[a] | No. of koalas | Lesion description[b] |
|---|---|---|
| Overt disease | Total: 12 | Conjunctivitis ($n$=8)<br>Microscopic findings: mild or moderate nonsuppurative or mixed hyperplastic conjunctivitis<br>Pericloacal staining ("wet bottom") ($n$=4) |
| Inapparent overt disease | Total: 29<br>Grossly evident internal lesions: 5<br>Histologically evident lesions only: 24 | Ocular lesions<br>Mild or rarely moderate nonsuppurative or mixed hyperplastic conjunctivitis ($n$=14)<br>Mild chronic active superficial keratitis ($n$=1)<br>Moderate focal suppurative and exudative hyperkeratotic dermatitis (eyelid) ($n$=1)<br>Urogenital lesions<br>Mild or rarely moderate nonsuppurative or mixed cystitis ($n$=19)<br>Penile and/or prostatic urethritis ($n$=9)<br>Mild and nonsuppurative or moderate nonsuppurative and rarely neutrophilic±lymphoid follicle formation<br>Paraovarian cyst ($n$=1)<br>Moderate mixed hyperplastic and ulcerative vaginitis with lymphoid follicle formation ($n$=2)<br>Mild nonsuppurative endometritis ($n$=1)<br>Mild multifocal nonsuppurative orchitis ($n$=2) |
| No detectable lesions | Total: 16 (2 females, 14 males) | |

[a] Overall classification of disease following Wan et al. (2011) and Polkinghorne et al. (2013).

[b] Individual koalas may have been presented with lesions at more than one site.

(80%) females. Of the 37 positive males, 33 (89%) had positive ocular swabs and 25 (68%) had positive urogenital swabs. Of the 20 positive females, 17 (85%) had positive ocular swabs and 15 (75%) had positive urogenital swabs. There was no statistical association between sex and site for PCR-positive swabs. All koalas in TWC II, III, and V were PCR positive (22/22, 10/10 and 2/2 respectively). In TWC I, 80% of koalas were affected (12/15), 75% were affected in TWC IV (9/12), and 50% in TWC VI (2/4). There was a relatively even distribution of cases between the five BCS categories. There were no statistically significant associations of *C. pecorum* infection status with either age or BCS of koalas.

#### Pathologic findings

The most common cause of death or reason for euthanasia was trauma attributed to motor vehicle or dog bite ($n$=30). Oxalate nephrosis (Speight et al. 2013) was the most common gross pathologic finding at necropsy ($n$=18).

Lesions consistent with chlamydial infection were identified in 41/57 (72%) koalas that were PCR positive for *C. pecorum* (Table 1). Gross lesions consistent with *Chlamydia* infection were observed in 17 *C. pecorum*–positive koalas, but overt clinical disease was present in only 12 (21%) koalas, as conjunctivitis ($n$=8) and brown staining around the cloaca ($n$=4). In the other five koalas, gross lesions were externally inapparent as they affected the urogenital tract. Another 24 *C. pecorum*–positive koalas had lesions detectable by histopathology only (Table 1), giving a total of 29 koalas (51%) with inapparent overt disease. Chronic or chronic–active conjunctivitis, cystitis, and urethritis were the most commonly found microscopic lesions consistent with chlamydiosis (Table 1).

Inapparent overt lesions suggestive of chlamydial infection included cystitis and reproductive tract lesions such as paraovarian

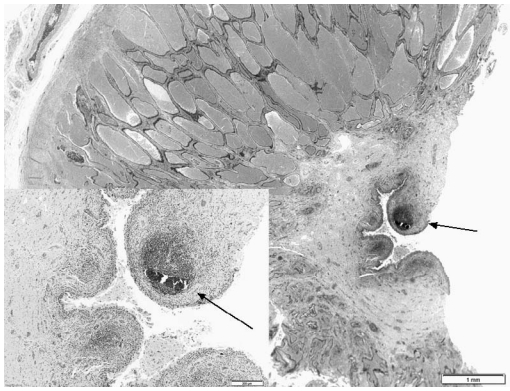

FIGURE 1. Prostate gland of a koala (*Phascolarctos cinereus*) from South Australia, Australia, found infected with *Chlamydia pecorum*. Periurethral subepithelial connective tissue is infiltrated by lymphocytes and plasma cells with formation of lymphoid follicles. Arrows, H&E stain. 2× objective. Bar=1 mm. Inset shows higher magnification of periurethral inflammatory infiltrates and lymphoid follicles. Arrows, H&E stain. 10× objective.

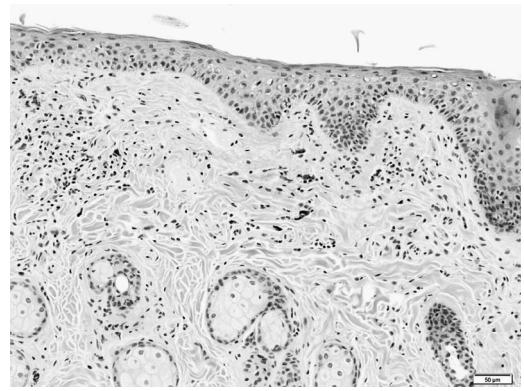

FIGURE 2. Eyelid of a koala (*Phascolarctos cinereus*) from South Australia, Australia, found infected with *Chlamydia pecorum*. Mild nonsuppurative inflammation in the superficial dermis surrounding blood vessels, scattered pigment (melanin)-laden macrophages in the dermis (pigmentary incontinence), and mild irregular epithelial hyperplasia and spongiosis at leading edge of the eyelid. H&E stain. 20× objective. Bar=50 μm.

cysts and endometritis, vaginitis, prostatitis, orchitis, and penile and prostatic urethritis (Fig. 1). Histopathologic inflammatory lesions in ocular and urogenital tissues were characterized by mild or less commonly moderate subepithelial and interstitial infiltrates of lymphocytes and plasma cells, with variable neutrophilic and histiocytic infiltrates. Conjunctivitis was characterized by mild or moderate nonsuppurative or mixed hyperplastic conjunctivitis with variable pigmentary incontinence (Fig. 2). Formation of lymphoid follicles was occasionally observed in penile and prostatic urethritis (Fig. 1) and mucosal and epithelial erosion and ulceration was a feature of some lesions. Paraovarian cysts were characterized by multiple thin-walled fine fibrocollagenous cysts lined by low cuboidal to flattened epithelium with infrequent papilliform projections of dense fibrovascular connective tissue covered by cuboidal epithelium.

Sixteen (28%) *C. pecorum*–positive koalas (2 females, 14 males) had no lesions suggestive of chlamydial infection, revealing a high subclinical carrier status, which was biased toward male koalas (14/16, 87.5%). Seven of the 65 koalas examined showed gross ($n$=4) or microscopic ($n$=3) ocular and urogenital lesions suggestive of *Chlamydia* infection but were PCR negative for *C. pecorum*.

## DISCUSSION

The 88% prevalence of *C. pecorum* infection was much higher than expected in this subset of the SA koala population and is likely due to the high number of koalas with subclinical or inapparent infections and the low numbers with clinically overt lesions. The majority of gross lesions were clinically inapparent, only detectable by post-mortem or microscopic examination, which further supports PCR as the most sensitive method of detection of chlamydial infection (Wan et al. 2011; Polkinghorne et al. 2013). These findings highlight that molecular testing should be used in combination with veterinary assessment to accurately diagnose chlamydial infection in koalas.

The most common lesions in SA koalas with chlamydial disease were conjunctivitis, cystitis, and urethritis, characterized by mild or moderate nonsuppurative (chronic) or mixed (chronic–active) inflammation, consistent with lesions reported in koalas elsewhere (Obendorf and Handasyde 1990; Hemsley and

Canfield 1997). Endometritis, vaginitis, ovarian cysts, and prostatitis, however, were rare findings in SA koalas. This differs from the presentation of chlamydial disease in koala populations along the eastern seaboard of Australia, in which chronic inflammation of the reproductive tract is a common and important presentation of chlamydiosis (Obendorf 1988; Canfield 1989; Obendorf and Handasyde 1990; Canfield and Spencer 1993; Hemsley and Canfield 1997; Higgins et al. 2005a). This lack of reproductive disease may help explain the abundance of the Mount Lofty koala population. Chlamydial infection also plays a role in pyogranulomatous pyelonephritis in at least some koala populations in New South Wales (Higgins et al. 2005b); however, this lesion was not observed in SA koalas.

The finding that *C. pecorum* was frequently detected in male koalas without lesions suggests subclinically infected males may be a relatively cryptic and important source of infection for naïve koalas, not readily detectable by routine clinical and pathologic examination. However, overall we found that males and females were equally infected. Because the main methods of transmission are proposed to be fighting and sexual activity (Whittington 2001), the high prevalence of infections in young TWC I koalas may be explained by the theory that infected dams can transmit *Chlamydia* to their juveniles by close contact or pap feeding, or during birth (Whittington 2001; Polkinghorne et al. 2013).

The finding of seven PCR-negative koalas with signs of disease suggests that other etiologies may underlie these lesions in affected koalas. *Chlamydia pneumoniae* infection is one possibility. In addition, Patterson et al. (2015) identified koalas with "wet bottom" in a population free of *C. pecorum* and *C. pneumoniae* by PCR testing of urogenital and conjunctival swabs, suggesting another significant cause (or causes) of wet bottom may exist.

Contradictory to long-held beliefs, we found a significant level of chlamydial infection in SA koalas; however, infection did not always result in clinical disease. This differs from *C. pecorum* infection in koala populations along the east coast of Australia, where a high prevalence of disease is associated with infection in some populations (Polkinghorne et al. 2013). Although we isolated *C. pecorum* from both urogenital and conjunctival swabs, a recent survey of 288 Victorian koalas from three mainland and island populations identified C. *pecorum* in affected populations from urogenital swabs only (Patterson et al. 2015). The differing expression of infection in the SA population is interesting, given that founding koalas of the Mount Lofty population were from Victorian populations (Robinson 1978). Difference in disease manifestation may be due to genetics, environmental stressors (e.g., urban habitat and human impact), or concurrent disease in the relatively isolated SA population. Genetic analysis of a small selection of koala *C. pecorum* strains from SA koalas suggested that these strains are not dissimilar to strains in other populations (Bachmann et al. 2014). However, detailed comparative genomic analyses are needed to explore this further.

This study of the occurrence and pathologic effects of *C. pecorum* in SA koalas provides valuable new information on the *Chlamydia* infection status of animals from this understudied region and further highlights the potential risk that these infections have on the long-term survival of koalas in SA and nationwide.

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