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Molecular Evidence for Subclinical Infection of *Chlamydia pecorum* in Captive Koalas (*Phascolarctos Cinereus*) of Japanese Zoos

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Abstract

Chlamydial infections pose a significant threat to the health and long-term survival of koalas. However, the pathogenesis of this disease is not well understood. *Chlamydia pecorum* (*C. pecorum*) is an important pathogen affecting wild koalas' health and their survival. However, the infection status of *C. pecorum* remains largely unknown in captive koalas across Japanese zoos. Therefore, in this study, we aimed to determine the infection status of *C. pecorum* in koalas from seven Japanese zoos. We tested swab samples obtained from koalas of these zoos for *C. pecorum* using qPCR and conventional PCR. We found a 21.74% overall prevalence of *C. pecorum* in these koalas. Female koalas showed a higher prevalence (24.24%) than male koalas (15.38%). However, no direct association was observed between koala health and *C. pecorum* loads. Notably, among *C. pecorum* positive koalas, some showed higher chlamydial loads without any classic signs associated with the progression of chlamydial diseases, such as ocular inflammation and wet bottom. We found higher *C. pecorum* load in adult koala than other groups. These results shed light on the subclinical infection of *C. pecorum* in captive koalas, and also may act as a guide for future control measures.

Keywords: Koala; Chlamydia; Prevalence; Japanese Zoo

Introduction

The koala (*Phascolarctos cinereus*) faces many threats to its survival as a species, and is experiencing population declines ranging from severe to catastrophic [1]. This iconic marsupial species has been placed on the International Union for Conservation of Nature (IUCN)'s Red List of Threatened Species [2]. Anthropogenic factors—such as habitat loss due to urbanization—and climate change are clearly major contributors to the decline in koala populations, but infectious disease may also play a role. Specifically, chlamydiosis has attracted much attention from conservationists as a disease with a potentially adverse impact [3,4].

In koalas, chlamydiosis can develop following infection with either of two chlamydiae species, *Chlamydia pecorum* (*C. pecorum*) or *Chlamydia pneumoniae* (*C. pneumoniae*) [5]. Of these species, *C. pecorum* is regarded as the more pathogenic and to be the causative agent for more severe disease [6]. *C. pecorum* infection in koalas may cause conjunctivitis, leading to blindness [7,8]. Other manifestations include urogenital changes such as urethritis, ureteritis, nephritis, cystitis, prostatitis, orchitis and epididymitis [8,9]. Moreover, *C. pecorum* infection has been reported to cause infertility in female koalas, resulting females lacking pouch youngs [10,11].

Despite the reports of asymptomatic chlamydial infection or limited overt diseases in chlamydial infection [12,13], the influence of chlamydial infection on long-term koala survival cannot be overlooked [12-14]. Certain *C. pecorum* genotypes have been associated with disease progression, while others have been associated with the absence of clinical disease [15]. The prevalence of *C. pecorum* infection in free range koala populations varies from region to region. Reported prevalence in populations and subpopulations across Australia include figures of 4% to 71% [4], 0%-85% [6,16,17], and up to 88% [18-20]. Some island populations are regarded as chlamydia-free, but the disease is generally regarded as endemic in mainland Australia, although there are differences in the manifestation of symptoms among and between populations with a high prevalence of *C. pecorum* [20].

In addition to the wild populations in Australia, zoos around the world are home to captive koala populations. These captive populations represent an invaluable source of genetic diversity and a bulwark against the threat to this species, as well as providing many opportunities for research on koala chlamydiosis and strategies to deal with it. Furthermore, the challenge of maintaining sustainable captive populations requires the diagnosis and treatment of the disease in zoo-reared koalas [21,22]. However, chlamydial disease in captive koalas remains less studied. For a better understanding of the influence of any disease on its host, the prevalence study is critical. Moreover, knowledge of prevalence and clinical severity could inform strategies to combat chlamydiosis. Therefore, it is important to know the prevalence of chlamydial infection status such as *C. pecorum* infection in the koalas both in the wild and captivity.

The koala population in Japanese zoos represents a promising target for research in this field. This population is maintained across 7 zoos in the country [23], with social housing at varying degrees of population density. These koalas receive regular medical examinations, and the zoos are affiliated with laboratories capable of PCR testing for the pathogens that cause chlamydial disease. In this study, we determined the prevalence of *C. pecorum* infection in koalas in Japanese zoos, through qPCR and conventional PCR analyses of ocular swab samples. Furthermore, we investigated any associations between *C. pecorum* infection and demographic factors such as age and sex and health parameters, including body condition and presence or absence of abnormal clinical signs.

Materials and Methods

Ethics approval and Consent to Participate

This study was performed in accordance with the protocols of the Institutional Animal Care and Use Committee of the Joint Faculty of Veterinary Medicine, Kagoshima University, Japan for scientific purposes (Approval Number: 19K001).

Sample Collection and Processing

We targeted the captive koala population in Japan for analysis. This population is housed in seven zoos across the country: Kanazawa Zoo, Tama Zoological Park, Kobe Oji Zoo, Awaji Farm England Hill Zoo, Hirakawa Zoological Park, Saitama Children's Zoo and Nagoya Higashiyama Zoo. All koalas' origin is Australia and most of them are from Queensland, except Awaji Farm England Hill Zoo in which koalas are originally from Victoria, Australia.

At each zoo, koalas were housed in environments maintained within a constant temperature range (23~25°C) using an air conditioning system. Each habitat is designed, as far as possible, to simulate the natural habitat in order to avoid any general stress, and provided the koalas with an ad libitum supply of eucalyptus leaves.

Between June and October 2021, ocular swab samples (n=46) were collected from the conjunctivas of both eyes

of each koala by zoo veterinarians in accordance with the relevant institution's standard operating procedure. The samples were then shipped to Transboundary Animal Diseases (TAD) Center (Kagoshima University, Japan) on ice, and stored at -30°C until analysis.

Demographic and Clinical Assessments

The sex and age of koalas were recorded from the zoo's data at the time of sampling. The koalas were subdivided into five age groups such as joeys (age <1 year), juvenile (age = 1-2 years), young adult (age = 2-4 years), adult (age = 4-8 years), old (age>8 years) koalas [24]. At the same time, each koala was examined by a zoo veterinarian to determine body condition score on a scale of 1 to 5. This score was determined by palpating muscle in the scapular region. The koala's condition was regarded as 'poor' based on a score ≤2, 'good' based on a score of 3, and 'very good" based on a score 5 [24]. Each koala was also observed for any visible clinical signs and subjected to blood sampling for determination of white blood cells (WBCs) count. Based on the results for body condition score, observed clinical signs, and WBC data, the zoo veterinarian made a comment on overall health status of individual koala.

Extraction of DNA

DNA was extracted from each ocular swab sample (400µL) using a QIAmp DNA Mini Kit, in accordance with the manufacturer's instructions (QIAGEN), and eluted in 100 µl of the AE Elution buffer provided with the kit. The DNA concentration and purity of the extracted DNA were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Waltham, MA USA), and DNA samples were kept at 30 °C until analysis.

Determination of *C. pecorum* by Real Time PCR

Quantitative real-time PCR (qPCR) was performed to test for the presence of *C. pecorum* and determine the chlamydial load in each koala, using extracted DNA as a template. The forward primer was 5' ATCGGGACCTTCTCATCGAG 3' and the reverse primer was 5' GACTAACAGTATAAGCAGTG 3', and the primers targeted a 108 bp fragment of the K7 gene for hypothetical protein gene partial coding sequence (LC681833.1) or partial genome sequence (CP088917.1, CP085634.1, CP080403.1 etc.) of *C. pecorum*. The qPCR assays were carried out with a total volume of 20µL, consisting of 10 µL Brilliant-III Ultra-Fast SYBR Green Q-PCR Master Mix (Agilent Technologies, Santa Clara, CA, USA), 0.2 µL of 10 pM forward and reverse primers and 5 µL DNA sample as a template, following the manufacturer's instructions using a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). PCR conditions were initial denaturation at 95°C for 3 min, and 40 cycles of 95°C for 5 s and primers annealing at 50°C for 10 s. The specificity of the PCR was confirmed by melt curve analysis. Samples were run in duplicate, and a positive and negative control, as well as a standard curve for the *C. pecorum* gene was included in all qPCR assays. All samples that showed ≥ 50 copies/µL DNA were considered as positive [17].

Determination of *C. pecorum* By Conventional PCR

A Phusion PCR was performed on a sub-set (10/46) of *C. pecorum* positive koalas for the validation of qPCR positive samples. *C. pecorum* positive DNA, as determined by qPCR was used as the template for conventional PCR amplification. The primers and their concentration used were the same as of qPCR (mentioned above). The cyclic conditions for the PCR were as follows: initial denaturation at 98°C for 2 min, denaturation at 98°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 1 min, followed by 40 cycles, and final extension at 72°C for 5 min. Next, the resulting PCR fragments were subcloned into the Zero Blunt TOPO vector and sequenced. The sequence data of *C. pecorum* were submitted to DDBJ (accession no. LC681833- LC681840).

Statistical Analysis

All statistical analyses were performed using the STATA software package, and p-values of ≤0.05 were regarded as significant. The Kruskal-Wallis test was performed to determine associations between *C. pecorum* load and risk factors based on demographic or health characteristics, age and body condition (BC). A Mann-Whitney test was also performed comparing between sex.

Results

Population, Clinical Signs and Health Status of Koala

Across the seven Japanese zoos, we obtained a total of 46 captive koalas for evaluation in this study. The age, sex, and body condition score of the study population are summarized in Table 1. This population comprised of 13 male (age range: 11 months to 12 years) and 33 female (age range: 10 months to 24 years) koalas. Results of clinical signs and WBC counts are shown in Table 2. Five of the 46 koalas showed some symptoms at sampling; specifically, loose stools in two koalas, alopecia in two koalas, and abdominal bloating in one koala. WBC counts in all koalas were normal [25]. In each case, the examining veterinarian made an overall comment on koala health as 'satisfactory' (Table 2).

Variables	Name of the Zoos						
	Kanazawa Zoo (n=4)	Tama Zoological Park (n=3)	Kobe Oji Zoo (n=8)	Awaji Farm England Hill Zoo (n=4)	Hirakawa Zoological Park (n=12)	Saitama Children's Zoo (n=5)	Nagoya Higashiyama Zoo (n=10)
Sex							
Male	1 (25%)	2 (66.67%)	2 (25%)	2 (50%)	2 (16.67%)	1 (20%)	3 (30%)
Female	3 (75%)	1 (33.33%)	6 (75%)	2 (50%)	10 (83.33%)	4 (80%)	7 (70%)
Age group							
Joey	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (20%)
Juvenile	1 (25%)	0 (0%)	0 (0%)	0 (0%)	5 (41.67%)	2 (40%)	1 (10%)
Young Adult	0 (0%)	1 (33.33%)	4 (50%)	0 (0%)	2 (16.67%)	2 (40%)	3 (30%)
Adult	3 (75%)	2 (66.67%)	3 (37.5%)	0 (0%)	4 (33.33%)	1 (20%)	1 (10%)
Old	0 (0%)	0 (0%)	1 (12.5%)	4 (100%)	1 (8.33%)	0 (0%)	3 (30%)
BC							
Poor	0 (0%)	0 (0%)	0 (0%)	1 (25%)	1 (8.33%)	0 (0%)	0 (0%)
Good	3 (75%)	3 (100%)	0 (0%)	3 (75%)	4 (33.33%)	5 (100%)	10 (100%)
Very good	1 (25%)	0 (0%)	8 (100%)	0 (0%)	7 (58.33%)	0 (0%)	0 (0%)

Table 1: Study population and characteristics (sex, age and body condition) of koalas from different zoos.

Koala ID	Sex	Age	Group	BC	*C. pecorum* copies/µl DNA	WBC (10^2/µl)	Clinical signs
KU_TZ_07	Female	3 Y	Young adult	Good	65.46	-	-
KU_HZ_19	Female	1 Y	Juvenile	Very good	3.3e+07	62	-
KU_HZ_20	Female	6 Y	Adult	Poor	79.48	69	Alopecia
KU_HZ_23	Female	4 Y 2 M	Adult	Very good	2684279	89	-
KU_HZ_25	Female	3 Y	Young adult	Good	64.08	81	Loose stool
KU_HZ_27	Female	1 Y	Juvenile	Very good	120.71	82	-
KU_HZ_28	Female	1 Y	Juvenile	Very good	1547.72	88	-
KU_NZ_37	Male	4 Y	Young adult	Good	69.25	85	-
KU_NZ_43	Female	1 Y	Juvenile	Good	443.32	69	-
KU_NZ_44	Male	11 M	Joey	Good	2417.08	69	-

Table 2: A Summary of *C. pecorum* infected koalas in this study.

Prevalence of *C. pecorum*

A quantitative PCR assay was performed using a *C. pecorum*-specific primer sets to determine infection status of captive koalas. The specificity (E=96.6%, R2=0.996, slope= 3.407) and melt curve of qPCR are shown in a supplementary figure (Fig S1). A total of 10 koalas were positive for *C. pecorum* across the seven zoos, with varied amount of DNA copies (Table 2). The overall prevalence of *C. pecorum* in this population was 21.74% (10/46). The highest prevalence of *C. pecorum* in koalas was in the Hirakawa Zoological Park (50%, 6/12), followed by Tama Zoological Park (33%, 1/3) and Nagoya Higashiyama Zoo (30%, 3/10), and variations between zoos were statistically insignificant (Figure S1).

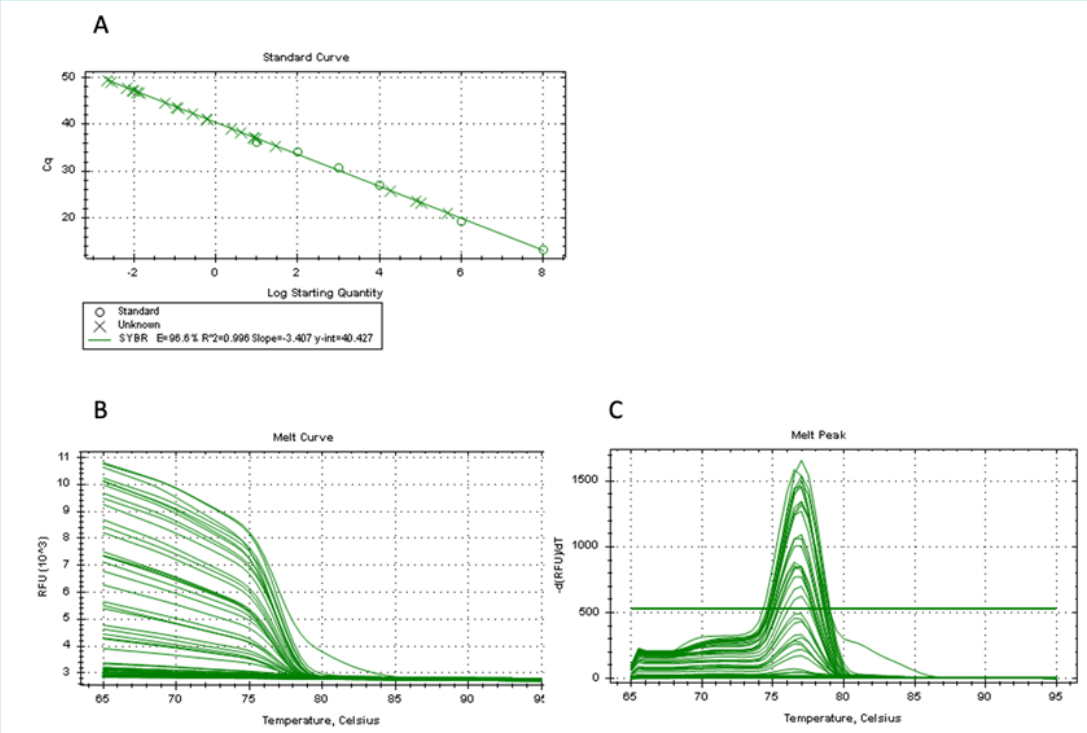

Figure-S1: The specificity of qPCR reaction. (A) A standard curve analysis, (B) A melt curve analysis, and (C) a melt peak are shown.

Variables	Prevalence (%) of *C. pecorum*	P value (Fisher's exact test)
Management unit		
Kanazawa Zoo	0/4 (0%)	0.065
Tama Zoological Park	1/3 (33%)	
Kobe Oji Zoo	0/8 (0%)	
Awaji Farm England Hill Zoo	0/4 (0%)	
Hirakawa Zoological Park	6/12 (50%)	
Saitama Children's Zoo	0/5 (0%)	
Nagoya Higashiyama Zoo	3/10 (30%)	
Sex		
Male	2/13 (15.38%)	0.411
Female	8/33 (24.24%)	
BC		
Poor	1/2 (50%)	0.109
Good	5/28 (17.86)%	
Very good	4/16 (25%)	
Age group		
Joey	1/2 (50%)	0.115
Juvenile	4/9 (44.44%)	
Young Adult	3/12 (25%)	
Adult	2/14 (14.29%)	
Old	0/9 (0%)	

Table 3: Prevalence of *C. pecorum* infection regarding zoo, sex, body condition, and age.

The *C. pecorum* load ranged between 69.25 and 2417.08 copies/µL DNA (median 1243.165) in infected male koalas, and 65.46 to 32807365.86 copies/µL DNA (median 282.015) in infected female koalas (Table 2). Female koalas also showed a higher prevalence than males (24.24%; Table 3). In addition, koalas with poor BC showed the highest prevalence of *C. pecorum* (50%; Table 3). By age, the highest prevalence of *C. pecorum* was found in joey (50%), followed by the juvenile (44.44%), adolescent (25%), and fully adult (14.29%) (Table 3).

Association between *C. pecorum* Loads And Population Characteristics

Mann-Whitney U test was performed for the detection of association between *C. pecorum* loads and population characteristics sex. The Kruskal-Wallis test also was performed to determine associations between *C. pecorum* load and risk factors based on age and BC. *C. pecorum* loads did not differ significantly with respect to sex (Figure 1A); however, the highest *C. pecorum* loads were observed in female koalas. Similarly, risk analysis revealed that BC and age cohorts were not significantly associated with *C. pecorum* load (Figures 1B-C).

However, we found higher *C. pecorum* load in very good BC koala followed by good and poor body condition koala. We also found higher *C. pecorum* load in adult koala, followed by juvenile and young adults.

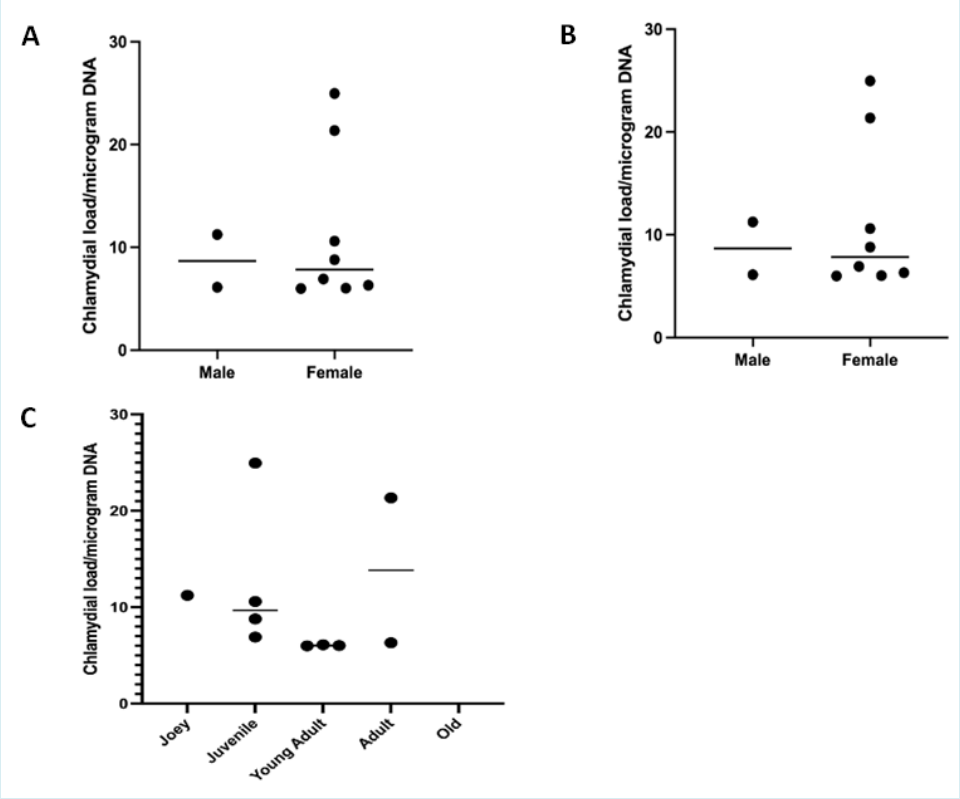

Figure 1: Associations of *C. pecorum* load with sex, BC, and age. *C. pecorum* load is indicated for sex (A) body condition, (B), and age (C). All resultant copy numbers have been log2 transformed. Each dot denotes an individual's *C. pecorum* load with median line displayed. Y-axis shows individual koala values (log transformed). For Statistical significance was evaluated using the Mann-Whitney U test and Kruskal-Wallis test. Data are presented as median bar.

Validation of *C. pecorum* Positivity by Conventional PCR

Ten koalas were found positive for *C. pecorum* by qPCR. Among 10 koalas 5 showed lower copy numbers than the lowest standard (10 copies) in the qPCR. Therefore, a conventional PCR was performed for further confirmation of *C. pecorum* gene, resulted 8 koalas positive for *C. pecorum*. After sub-cloning and sequencing, we found the target size (108 bp) of the DNA fragment (Figure 2), and was 100% homogenous with NCBI reference sequences (CP088917.1, CP085634.1, CP080403.1, CP080401.1, CP004035.1, CP004034.1, CP004033.1, and CP002608.1).

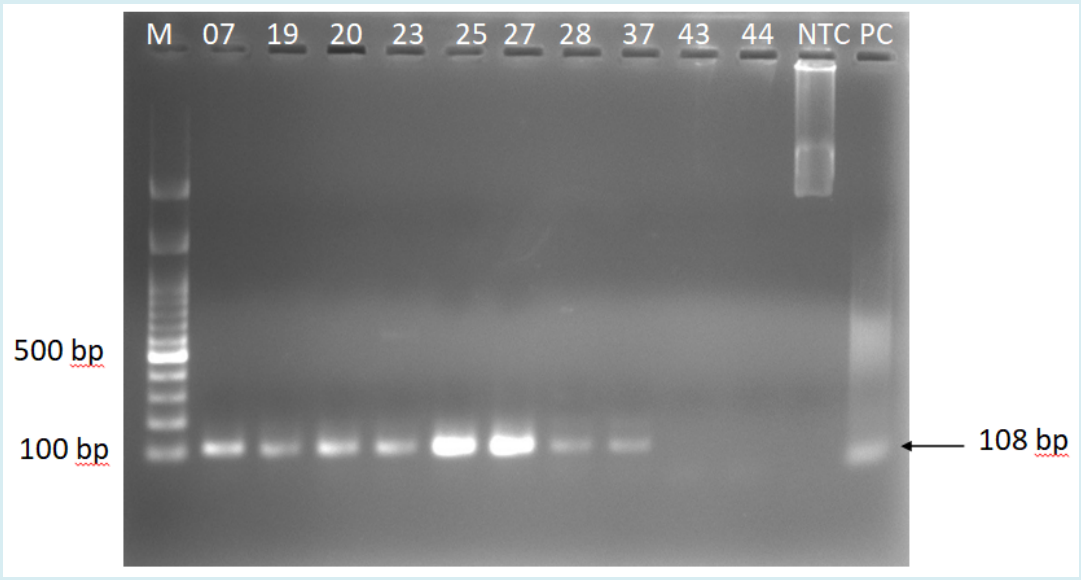

Figure 2: Amplification of *C. pecorum* gene using genomic DNA from the conjunctival swab samples of koalas. Lane M, 100 bp DNA marker (GeneDireX, Inc.); lane (07=KU_TZ_07, 19=KU_HZ_19, 20=KU_HZ_20, 23=KU_HZ_23, 25=KU_HZ_25, 27=KU_HZ_27, 28=KU_HZ_28, 37=KU_NZ_37, 43=KU_NZ_43, 44=KU_NZ_44)
Samples; Lane NTC, no-template control; Lane PC, positive control.

Discussion

To the authors' knowledge, this is the first report on *C. pecorum* (one of the important causative agents for chlamydiosis) prevalence in the captive koala population throughout the Japan. We determined the *C. pecorum* prevalence based on qPCR and conventional PCR analyses of conjunctival swab samples collected from the koalas at each of seven Japanese zoos. We also determined the chlamydial load in each koala, and evaluated associations with demographic and health parameters to provide useful information on chlamydia infection and disease in captive koalas.

As a major novel finding in this study regarding prevalence, we found 21.74% of the captive koalas in Japanese zoos had subclinical infection with *C. pecorum*. This figure partially reflects the prevalence reported in wild koala populations [16,26,27], in line with the figures of 25% and 41% reported for wild koalas in New South Wales and Queensland, respectively [18], but lower than the figure of 88% for South Australia [20]. However, this prevalence was not uniform across the Japanese zoos, and ranged from 0% to 50%. Thus, further monitoring of the Japanese captive koala population may generate useful data to formulate decision on how to best conserve wild koala populations in Australia. The highest prevalence among Japanese zoos was noted at the zoo with the largest koala population (Hirakawa Zoological Park, Kagoshima), and this result could be consistent with poorer health of koala. Also, relatively high *C. pecorum* positive ratio was observed in female (Table 1) possibly due to the group rearing of female koalas in Japanese zoo.

No significant association was observed between chlamydial load, age, sex, and zoo. Moreover, the lack of any association between chlamydial load and overt signs of disease in the study population provides some interesting evidence for the debate over the severity of chlamydiosis as a threat to wild koalas, warranting further investigation on the pathogenicity of *C. pecorum* in the wild koala populations [16,26,27].

Researcher has argued that *C. pecorum* of itself may not be the major driver of population decline for the species. Indeed, KoRV-B has been reported to be a significant contributing factor to the development of chlamydial disease [28]. The clinical onset of chlamydiosis may be triggered by factors such as environmental stress, such as anthropogenic habitat destruction [29]. This might explain associations between *C. pecorum* infection and clinical disease in the wild, but the infection would not be the causative factor [13]. In our study, the higher prevalence was observed in joey, followed by juveniles, and the low prevalence was seen in older koalas. In contrast, a previous study [30] reported the higher prevalence in juvenile koala. These age-related findings are also consistent with those reported in a previous epidemiolocal investigation in wild koalas [31]. The Japanese captive koala population is too small to allow a definitive conclusion, but the absence of overt disease among *C. pecorum*-positive koalas in this study may support

the argument that susceptibility to chlamydial disease may be linked to environmental stress, in a key finding from this study. However, further investigations of the pathogenesis of chlamydial disease are clearly warranted.

We found no significant association between sex and chlamydial load in *C. pecorum*-positive koalas, as stated above. However, prevalence appeared to be markedly higher in females than males (24.24% vs. 15.38%). Further studies in larger populations are required, but these findings tend to support the possibility that female koalas may act as subclinical carriers of *C. pecorum* infection. Couple with the high prevalence in juvenile koalas, our study findings may suggest that chlamydia can be transmitted from dams to their off-spring through close contact and pap feeding. Sex and fighting have been suggested as routes of transmission [26,32], but our findings suggest these routes may not have played a major role for infections in this study population.

Due to some limitations of handling of zoo koala, the study, chlamydial detection was based on ocular swab samples taken from the bilateral conjunctivas of each animal. In the majority of previous relevant studies, samples were obtained by swabbing of the urogenital tract or conjunctivas of free-ranging koalas. However, Canfield et al. reported 16% *C. psittaci* positivity in conjunctival swab samples obtained from captive koalas [33].

Non-endogenized KoRV subtypes have been suggested to comprise the health of koalas [34]. There may be other factors/agents that might contribute to *C. pecorum* infection in koalas, such as the presence of other chlamydial species, KoRV infection and/or other viral infections [30]. We observed that all koalas used in this study were infected with KoRV-A and some of them were co-infected with other KoRV subtypes [23]. The effect of KoRV subtype co-infection should be addressed in the future study. Overall, this study shows a subclinical chlamydial infection in these koala populations, and therefore it might be interesting to investigate further how/if chlamydial strain, koala genetics, stress levels and immunophenotype, co-infections by KoRV or herpesviruses, or stress responses might affect koala health.

Competing Interests

The authors declare that they have no competing financial interests.

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