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Genetic diversity of *Chlamydia pecorum* strains in wild koala locations across Australia and the implications for a recombinant *C. pecorum* major outer membrane protein based vaccine

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ABSTRACT

The long term survival of the koala (*Phascolarctos cinereus*) is at risk due to a range of threatening processes. A major contributing factor is disease caused by infection with *Chlamydia pecorum*, which has been detected in most mainland koala populations and is associated with ocular and genital tract infections. A critical aspect for the development of vaccines against koala chlamydial infections is a thorough understanding of the prevalence and strain diversity of *C. pecorum* infections across wild populations. In this study, we describe the largest survey (403 koalas from eight wild populations and three wildlife hospitals) examining the diversity of *C. pecorum* infections. 181 of the 403 koalas tested (45%) positive for *C. pecorum* by species-specific quantitative PCR with infection rates ranging from 20% to 61% in the eight wild populations sampled. The *ompA* gene, which encodes the chlamydial major outer membrane protein (MOMP), has been the major target of several chlamydial vaccines. Based on our analysis of the diversity of MOMP amino types in the infected koalas, we conclude that, (a) there exists significant diversity of *C. pecorum* strains in koalas, with 10 distinct, full length *C. pecorum* MOMP amino types identified in the 11 koala locations sampled, (b) despite this diversity, there are predicted T and B cell epitopes in both conserved as well as variable domains of MOMP which suggest cross-amino type immune responses, and (c) a recombinant MOMP-based vaccine consisting of MOMP "F" could potentially induce heterotypic protection against a range of *C. pecorum* strains.

1. Introduction

The koala (*Phascolarctos cinereus*) is an arboreal, folivorous marsupial native to Australia, and the only living representative of the family *Phascolarctidae*. Recently, the Australian Federal Government (April 2012) listed

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koala populations in Queensland, New South Wales and the Australian Capital Territory as "vulnerable". The decline across various populations in Australia, particularly in previously densely populated areas, has been attributed to several factors, including, habitat loss due to land clearing (Melzer et al., 2000), chlamydial disease (Jackson et al., 1999), motor vehicle trauma (Dique et al., 2003) and dog attacks (Lunney et al., 2007). Chlamydial infections are a major cause of disease in koalas (Polkinghorne et al., 2013). Two species, *Chlamydia pecorum* and *C. pneumoniae*, have been isolated from many koala populations, with *C. pecorum* being the most widespread and pathogenic of the two species (Jackson et al., 1999). The clinical manifestations of *C. pecorum* include keratoconjunctivitis (ocular disease) leading to blindness, as well as urinary and genital tract disease, resulting in inflammation and fibrosis of the bladder and the upper female genital tract (Blanshard and Bodley, 2008). The impact of these infections is further highlighted by a recent study which found that older koalas with clinical signs of chlamydiosis made up the most frequent admission group to a koala rehabilitation facility over 30 years (Griffith et al., 2013).

The gross and histological similarity between chlamydial lesions in koalas and those induced by *Chlamydia trachomatis* in humans (Hemsley and Canfield, 1997) is suggestive of similar disease pathologies. In humans, the diversity of the major outer membrane protein (MOMP), which is encoded by the *ompA* gene (Kaltenboeck et al., 1993), is an important factor in pathogenesis via immune evasion and subsequent infection by multiple MOMP serovars. MOMP has been considered a potential vaccine candidate for *C. trachomatis* in primate models (Kari et al., 2009) as well as *C. pecorum* in koalas (Kollipara et al., 2012, 2013). The strains of *C. trachomatis* have been divided into 15 serovars based on variations in the MOMP. This sequence variation is mainly limited to the four surface-exposed variable domains which are interspersed between five conserved domains. Originally, serovars were distinguished based on their recognition by patient sera (Wang et al., 1985), however the *ompA* sequences for each serovar have now been well characterised. Organisms assigned to a serovar group on the basis of their translated *ompA* sequences are referred to here as amino types/aminovars.

Outer membrane protein A (*ompA*) genotyping targeting variable domains 3 and 4 has been used previously to investigate *C. pecorum* infections in wild koala populations (Jackson et al., 1997), usually with the goal of better understanding *C. pecorum* transmission. However, the analysis of *ompA* sequence variation is also relevant to the utility of MOMP as a target for an effective *C. pecorum* vaccine. For such vaccine development, which will use full length MOMP protein, it is appropriate to sequence and analyse full length *ompA* from a range of *C. pecorum* isolates across the koala's natural habitat range. As such, the primary objectives of this study were to, (a) investigate the polyphyletic nature of full length *ompA* sequences in several *C. pecorum* wild koala populations and (b) examine the implications of MOMP diversity for developing an efficient *C. pecorum* vaccine for koalas.

2. Materials and methods

2.1. Koala samples

Two types of samples were collected from across the entire range of wild koalas. The first involved the sampling of wild koalas presenting for veterinary treatment because of injury or illness (termed wildlife hospital koalas) at (a) Tanilba Bay Veterinary Hospital, Port Stephens, New South Wales (NSW), (b) Port Macquarie Koala Hospital, Port Macquarie, NSW and (c) Adelaide Hills Animal Hospital, Stirling, South Australia (Fig. 1). At these facilities, swabs were collected as a part of routine testing for the care of diseased animals and their use in this study was approved by the Queensland University of Technology (QUT) Animal Ethics Committee and approved under Tissue Use Notification # 1100000718.

The second source of samples came from independent field sampling and investigations into the health of wild koala populations in Queensland (termed natural/wild koala populations). These locations included St Bees Island, North Stradbroke Island, Elanora, Narangba, Brendale, East Coomera and Lower Beechmont in Queensland and Byron Bay, NSW (Fig. 1). A standardised veterinary assessment of koalas was conducted on captured koalas and swab samples were taken from the conjunctiva, nasal cavity and urogenital sinus/cloaca (females), or from the urethra (males) and stored for later PCR screening.

2.2. Detection of C. pecorum infection using a specific PCR assay

All swab samples from koalas sampled as a part of independent field studies or collected as a part of routine veterinary treatment were screened for the presence of *C. pecorum* by a species-specific quantitative PCR (qPCR) targeting the 16S rRNA gene, as described in Marsh et al. (2011).

2.3. C. pecorum ompA sequencing

To evaluate the level of genetic diversity in the MOMP-encoding *ompA* gene from koala *C. pecorum* strains, *C. pecorum* positive DNA samples detected by our qPCR screening of koala swab samples was used a template for conventional PCR amplification of the near full length *ompA* gene (1140 bp) for each sample. Primers used in this reaction were *ompA*for (5′-ATGAAAAAACTCTTAAAATCGG-3′) and *ompA*rev (5′-TTAGAATCTGCATTGAGCAG-3′). PCR conditions were a single cycle of initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 54 °C for 40 s, primer extension at 72 °C for 90 s, followed by a final extension at 72 °C for 7 min. *ompA* sequences were determined by direct sequencing of the PCR product using *ompA*for/*ompA*rev following the use of a BigDyeTerminator 3.1 Sequencing kit (Life Technologies, Victoria, Australia) and subsequent purification according to the manufacturer's instructions. Dideoxy sequencing was performed at the QUT DNA sequencing facilities, using an Applied Biosystems ABI3500 Gene analyser.