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Within-population diversity of koala *Chlamydophila pecorum* at *ompA* VD1-VD3 and the *ORF663* hypothetical gene

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ABSTRACT

Infection of koalas by *Chlamydophila pecorum* is very common and causes significant morbidity, infertility and mortality. Fundamental to management of the disease is an understanding of the importance of multi-serotype infection or pathogen virulence in pathogenesis; these may need consideration in plans involving koala movement, vaccination, or disease risk assessment. Here we describe diversity of *ompA* VD1-3, and *ORF663* hypothetical gene tandem repeat regions, in a single population of koalas with diverse disease outcomes. We PCR amplified and sequenced 72 partial *ompA* segments and amplified 25 tandem repeat segments (*ORF663* hypothetical gene) from *C. pecorum* obtained from 62 koalas. Although several *ompA* genotypes were identified nationally, only one *ompA* genotype existed within the population studied, indicating that severe chlamydial disease occurs commonly in free-ranging koalas in the absence of infection by multiple MOMP serotypes of *C. pecorum*. In contrast, variation in tandem repeats within the *ORF663* hypothetical gene was very high, approaching the entire range reported for pathogenic and non-pathogenic *C. pecorum* of European ruminants; providing an impetus for further investigation of this as a potential virulence trait.

1. Introduction

Chlamydophila pecorum and *Chlamydophila pneumoniae* both occur in koalas. Chlamydial inclusions have been demonstrated in direct association with keratoconjunctivitis, and mild to severe inflammation and fibrosis of the urinary bladder, prostate, kidney, uterus and fallopian tubes (Hemsley and Canfield, 1997; Higgins et al., 2005a). Though studies that compare relative prevalence and pathogenicity of *C. pecorum* and *C. pneumoniae* in koalas (Jackson et al., 1999; Griffith, 2010) are limited by sample size and restricted location, they suggest that *C. pecorum* is more commonly associated with urogenital disease and severe keratoconjunctivitis, while *C. pneumoniae* is more commonly associated with mild ocular and respiratory signs.

The gross and histological similarity between chlamydial lesions in koalas and those induced by *Chlamydia trachomatis* in people (Hemsley and Canfield, 1997) suggest the possibility of a similar pathogenesis. In humans, diversity of the major outer membrane protein (MOMP), which is encoded by the *ompA* gene and has four variable domains (VD1-4) (Kaltenboeck et al., 1993), is a potentially important factor in pathogenesis by permitting immune evasion and subsequent infection by multiple MOMP serotypes. Resultant repeated exposure to conserved antigens such as hsp60 induces severe inflammatory damage in the host (Morrison, 1991). It appears possible that this mechanism may also play a role in chlamydial disease in koalas; five *ompA* VD4 genotypes have been identified among 15 isolates of koala *C. pecorum* across Australia (Jackson *et al.*, 1997) and chlamydial tubal infertility in koalas is associated with high titres of antibody against hsp60 (Higgins et al., 2005b). This

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mechanism would require within-population diversity of MOMP, but this has not yet been examined in any koala population.

Investigation of potential virulence genes of koala *C. pecorum* also requires investigation. One potential candidate, *ORF663* is a hypothetical gene that contains a variant tandem repeat of 15 nucleotides. Analysis of European ruminant *C. pecorum* strains suggested a lesser number of motif repeats was associated with pathogenic strains, relative to enteric strains but a larger sample size is required to confirm these findings (Yousef-Mohamad et al., 2008). Investigation of chlamydial virulence in free-ranging koalas is made difficult by the many factors confounding study of the disease state, such as chronicity, and host sex, age and nutritional status. Investment in such studies would need to be founded on knowledge of whether variation in tandem repeats within this hypothetical gene exists within a single population with diverse disease outcomes. This has not yet been investigated in koalas.

This paper reports the first investigation of the diversity of koala *C. pecorum* strains within a semi-urban koala population with diverse chlamydial disease outcomes (the Port Macquarie area of New South Wales) (Griffith, 2010), to determine whether chlamydial pathogenesis in koalas is dependent upon multiple MOMP-serotype infection, or whether diverse genotypes of the *C. pecorum ORF663* hypothetical gene exist within a single population of koalas.

2. Materials and methods

The study made use of randomly selected, archived urogenital and conjunctival swabs from hospitalized koalas of the Port Macquarie area; a 1500 km^2 area composed of several locales separated by roads, rivers and urban development, on the central coast of New South Wales, Australia (31° 26′ 0″ S, 152° 54′ 0″ E) (Appendix 1). Consistent with the population of koalas admitted to the hospital (Griffith, 2010), swabs comprised those from koalas with none, some or all of the signs associated with chlamydial disease: keratoconjunctivitis, urinary incontinence and paraovarian cysts. Swabs were stored at −20 °C until DNA was extracted using QuickExtract (Epicentre Biotechnologies, Madison, Wisconsin, USA), according to manufacturer's instructions. PCR reactions of 50 μL comprised 3 μL 1:20–1:100 extracted DNA, 200 μM dNTPs, 0.5 μM each primer, 10 μL 10× BSA, 3 mM $MgCl_2$, 5 IU Taq DNA polymerase (M0273 New England Biolabs Inc, MA). Sterile milliQ water was used as template in negative controls. Cycling conditions were: initial denaturation (94 °C, 5 min) followed by 40 cycles of denaturation (94 °C, 60 s), annealing (55 °C, 60 s) and extension (72 °C, 120 s), and then final extension (72 °C, 10 min) in an Eppendorf Mastercycler Gradient thermocycler. Primers CTU (5′-ATGAAAAAACTCTTGAAATCGG-3′) and CTL (5′-CAAGMTTTTCTAGAYTTCATYTTGTT-3′) were used, based on Denamur et al. (1991), in order to produce a fragment that covered three of the four variable domains, yet was of a length compatible with available commercial sequencing.

A sample (3 μL) of each PCR product was electrophoresed through a 1.5% agarose/TBE gel. Single products of 720 bp were purified with SAP/EXO1 exonuclease (Amersham Biosciences, Sweden; 37 °C, 30 min; 80 °C, 15 min; 15 °C, 10 min). Where multiple products were detected, the 720 bp fragment was excised and purified using a GFX gel band purification kit (GE Healthcare, Piscataway, NJ). Purified products were sequenced on forward and reverse strands using primers CTU and CTL (Macrogen, Seoul, South Korea). Sequences obtained from only one swab were confirmed by a second PCR, using a High Fidelity Taq polymerase (Platinum Taq, Invitrogen, Australia), and sequencing as above. GenBank accession numbers are presented in Appendix 1.

For comparison, existing sequences for European ruminant *C. pecorum* isolates were obtained from GenBank and additional sequences were generated, as described above, from diagnostic swab material obtained from two sheep isolates and several koala isolates from areas remote to Port Macquarie (Appendix 1). Sequence chromatograms were edited in Sequencher 4.8 (Gene Codes Corp.), and translated amino acid sequences were aligned with ClustalX (Thompson et al., 1997). Pal2Nal was then used to obtain a DNA-based alignment from the amino acid alignment (Suyama et al., 2006). Phylogenetic analyses were performed on individual and combined datasets using Bayesian Inference with the program MrBayes 3.0. Parameters for the selected model of substitution (general time reversible) were estimated from the data. A total of 30,000 trees were obtained (ngen = 3,000,000, samplefreq = 100), and the first 5000 of these were considered as the 'burn in' and discarded. A 50% majority-rule consensus tree of the remaining trees, including branch lengths (sumt) was produced.

A subset of isolates from the two most prevalent *ompA* genotypes, Ko5a and Ko5f (Appendix 1) were selected for amplification of the tandem repeat region of the *ORF663* hypothetical gene. Each 25 μL PCR reaction comprised 2.5 μL of 10× buffer, 200 μM each dNTP, 0.1 μL Taq DNA polymerase (HotStar Taq DNA polymerase, QIAGEN Pty Ltd, Doncaster, Australia), 1.5 μL 1:20 extracted DNA, and 0.5 μL each primer (5′-AAACAACTGCACCGCTCTCT-3′, 5′-CAAGGACTTTCTGGGGGAAG-3′) (Yousef-Mohamad et al., 2008). DNA extracted from a *C. pecorum* culture was used as template in positive controls and sterile milliQ in negative controls. Cycling conditions were: initial taq activation (95 °C, 15 min), followed by 35 cycles of denaturation (94 °C, 30 s), annealing (57 °C, 45 s) and extension (72 °C, 60 s), and then final extension (72 °C, 7 min), in a Bio-rad MJ Mini thermal cycler (Bio-rad Laboratories, Pty, Ltd, Gladesville, Australia). Products were electrophoresed in 1.5% agarose (Agarose I, Amresco Inc, Solon, USA) with GelRed™ fluorescent gel stain (Biotium, Inc, Hayward, USA) at approximately 10 V/cm of gel in TBE buffer for 1 h. Product fragment length was estimated against a standard ladder (Hyperladder™ IV, Bioline (Aust) Pty Ltd, Alexandria, Australia). Ten of the 25 products were purified, sequenced, edited and assembled as above, to confirm identity of the PCR product and to provide the information necessary to estimate the number of repeat motifs from the amplicon length of remaining products.