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Comparison of subcutaneous *versus* intranasal immunization of male koalas (*Phascolarctos cinereus*) for induction of mucosal and systemic immunity against *Chlamydia pecorum*

Courtney A. Waugh[a,b], Peter Timms[a,b], Dean Andrew[a,c], Galit Rawlinson[d], Jacqui Brumm[d], Karen Nilsson[d], Kenneth W. Beagley[a,*]

[a] Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Ave, Kelvin Grove 4059, Queensland, Australia
[b] Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, 90 Sippy Downs Dr, Sippy Downs 4558, Queensland, Australia
[c] Centre for Biomedical Research, Burnet Institute, 85 Commercial Road, Melbourne 3004, Victoria, Australia
[d] Lone Pine Koala Sanctuary, Fig tree Pocket, Brisbane 4519, Queensland, Australia

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ABSTRACT

Chlamydia pecorum infections are debilitating in the koala, contributing significantly to morbidity and mortality, with current antibiotic treatments having minimal success and adversely affecting gut microflora. This, combined with the sometimes-asymptomatic nature of the infection, suggests that an efficacious anti-chlamydial vaccine is required to control chlamydial infections in the koala. To date vaccination studies have focused primarily on female koalas, however, given the physiological differences between male and female reproductive tracts, we tested the efficacy of a vaccine in 12 captive male koalas. We evaluated the potential of both subcutaneous and intranasal vaccine delivery to elicit mucosal immunity in male koalas. Our results showed that both intranasal and subcutaneous delivery of a vaccine consisting of *C. pecorum* major outer membrane protein (MOMP) and the adjuvant immunostimulating complex (ISC) induced significant immune responses in male koalas. Subcutaneous immunization elicited stronger cell-mediated responses in peripheral blood lymphocytes (PBL), and greater plasma antibody levels whereas the intranasal immunization elicited stronger humoral responses in urogenital tract (UGT) secretions. This is the first time a *Chlamydia* vaccine has been tested in the male koala and the first assessment of a mucosal vaccination route in this species. Our results suggest that vaccination of male koalas can elicit mucosal immunity and could contribute to the long-term survivability of wild populations of the koala.

1. Introduction

Chlamydia pecorum is the most important pathogen adversely affecting the health of wild koala populations on mainland Australia [1]. *C. pecorum* infections have devastating effects in the koala, including kerato-conjunctivitis, urinary tract disease and reproductive tract disease, which may lead to blindness, urinary incontinence, infertility, and in severe cases euthanasia and/or death [1,9–11]. Early stages of infection can be treated with antibiotics, however, though the currently accepted regime (60 mg/kg for 45 days of chloramphenicol) appears to control mild chlamydial infections and prevent shedding, it does not treat severe urogenital (UGT) tract disease, and it is not known if chloramphenicol prevents reoccurrence of infection following cessation of treatment [2]. Furthermore, many cases prove to be asymptomatic in nature and go untreated, such that some overtly healthy koalas are found to be shedding high loads of *Chlamydia* [9], and thus acting as reservoirs and contributing to the spread of the disease. What is clear is that antibiotics alone are not sufficient to affect a cure and/or maintain healthy populations. Recent modeling on a koala population in QLD has suggested that for a range of anti-chlamydial vaccine efficacy levels the population decline due to *Chlamydia* could be reversed in just 5 years, but a vaccination schedule will likely need to be ongoing to maintain low *Chlamydia* prevalence [12]. Thus, we propose that a vaccine will provide an important conservation management tool for this species.

A chlamydial vaccine, to be considered effective, should induce both cellular and humoral responses in the host. A prime antigen target in several vaccine studies is the Major Outer Membrane Protein (MOMP), which constitutes 60% of the chlamydial outer

* Corresponding author. Tel.: +61 7 3138 6195; fax: +61 7 3138 6030.
E-mail address: k2.beagley@qut.edu.au (K.W. Beagley).

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membrane [13–16]. However, to date *Chlamydia* vaccine studies have focused primarily on female animal models, and indeed initial efforts to develop a MOMP-based vaccine for the koala have been successful in inducing a cell-mediated response, as well as a humoral immune response, in the female koala following subcutaneous (s.c.) vaccination [4–7]. Further, this prototype vaccine has been shown to be safe, while eliciting a strong immune responses in healthy females, without worsening clinical symptoms in already diseased animals [4]. Importantly, recent trials have also shown cross-reactivity of MOMP antibody responses from vaccinated healthy and diseased koalas [4,6], which implies the potential for wide cross-protection against the variety of genetically distinct *C. pecorum* strains circulating in wild koala populations [17]. However, for optimum protection of wild koalas a chlamydial vaccine must be effective in both sexes, thus the obvious next step is to assess the vaccine efficacy in the male koala. Further, the route of administration can affect the effectiveness of a vaccine, and in the murine model generally an intranasal (i.n.) or intraurethral (i.u.) vaccine will elicit a greater response against a genital *Chlamydia* challenge than a systemic route [18–20]. An i.u. route is deemed too invasive for the koala, thus here we have explored i.n. and s.c. routes.

The aim of this study was to assess the immune response profiles of two vaccination protocols, comparing a mucosal (i.n.), and a systemic (s.c.) route of immunization in the male koala. To the best of our knowledge this is the first study where a *Chlamydia* vaccine has been tested in the male koala, and *via* a mucosal immunization route in the species.

2. Animals, materials and methods

2.1. Animals

Animals were recruited from a captive population at Lone Pine Koala Sanctuary (LPKS), Brisbane, Australia. Each animal was clinically healthy and *Chlamydia*-negative prior to vaccination. There were no natural chlamydial infections detected in the Lone Pink Koala population throughout the duration of the study. The koalas recruited into the study were kept in small groups of males in confined areas and were aged between 3 and 8 years old. Inclusion criteria stipulated that animals must be males of breeding age (>2 years old), clinically healthy, and had not been enrolled in, nor had a mother that had been enrolled in a previous vaccine trial. 12 koalas that met the aforementioned criteria were enrolled in the study and assigned at random to each group. All procedures were approved by the Queensland University of Technology (QUT) Animal Ethics Committee (approval number 1000000638).

2.2. Immunizations

To test differences in the immune response due to the route of administration 6 koalas received an intranasal (i.n.) version of the vaccine, and 6 received the vaccine *via* the sub-cutaneous (s.c.) route. Each koala received three immunizations at monthly intervals, and samples (whole blood, urogenital (UGT), and ocular swabs) were collected at 0, 6, 10, 24, and 52-week time points. Pre-immunized samples (week 0) were collected immediately preceding the initial immunization event.

The major antigenic component of the vaccine was recombinant chlamydial major outer membrane protein (rMOMP), encoded by the *ompA* gene, cloned from sequences amplified from a koala *C. pecorum* isolated from South-East Queensland, combined with immune stimulating complex adjuvant (ISC; Pfizer/Zoetis) [7]. Production of rMOMP proteins for vaccination and sample collection are described in detail elsewhere [7].

2.3. Lymphocyte proliferation assay

Lymphocyte proliferation assays were performed as per Carey et al. [7] except that UV-inactivated *C. pecorum* genotype G [21] EBs were used for *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs). Proliferation was expressed as the percentage of PBMCs that had undergone two or more cell divisions, discerned by the reduction in mean fluorescence intensity as detected by flow cytometry.

2.4. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISAs) were performed as per Carey et al. [7] except for using host specific *C. pecorum* MOMP as antigen.

2.5. In vitro neutralization

In vitro neutralization assays were performed on animals pre-immunization, and post immunization (10 and 52 weeks) according to Carey et al. [7] except for using host specific *C. pecorum* genotype G Elementary Bodies (EBs). Both cells and inclusions were counted under the microscope and neutralization percentage determined compared to media only, and pre-immunization controls. Pre-immunization plasma, ocular and UGT secretory samples were collected from each individual animal and were used to establish the background level for each koala. The individual background level was then subtracted from each post-vaccination level for each individual to correct for baseline levels.

2.6. Statistics

All statistical analysis was performed using Graph-Pad Prism version 6 (Graph Pad Software, La Jolla, CA, USA) and SPSS (v.21; IBM). Data did not pass the test for normality (D'Agostino & pearsons omnibus normality test; p value) alpha = 0.05. Therefore nonparametric related samples Friedman's two-way ANOVA was used to analyze within cohorts. Data between cohorts was analyzed using the Independent-Samples Mann–Whitney U test. The p value was set at $\leq$0.05. Data are presented as medians (Mdn) showing interquartile range (IQR).

3. Results

3.1. PBMC proliferation

The rMOMP-based vaccine produced antigen-specific PBMC proliferative response to intact EBs after immunization in both the s.c. and i.n. cohorts, which was maintained up to 52 weeks post-vaccination (Fig. 1). A Friedmans 2-way ANOVA demonstrated a significant difference over time in the s.c. group (X^2 (4) = 10.421, p = 0.034) but not in the i.n. group (X^2 (4) = 3.459, p = 0.0484). A Mann–Whitney U test was conducted that examined the effect of delivery route post-vaccination on proliferation response. This showed a statistically significant effect due to delivery route, at 6 weeks (U = 4.5, p = 0.016) and 52 weeks (U = 5, p = 0.017) with the s.c.-immunized koalas maintaining a stronger response throughout (Fig. 1).

3.2. MOMP antibody response

3.2.1. IgG antibody response in plasma of the male koalas post-vaccination

Vaccine induced antigen-specific Immunoglobulin G (IgG) antibodies were detected in plasma in both the i.n. and s.c. groups up until 52 weeks post-vaccination (Fig. 2A.). In the s.c. group