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Vaccination of koalas (*Phascolarctos cinereus*) with a recombinant chlamydial major outer membrane protein adjuvanted with poly I:C, a host defense peptide and polyphosphazine, elicits strong and long lasting cellular and humoral immune responses

Shahneaz Ali Khan[a], Courtney Waugh[a,b], Galit Rawlinson[c], Jacqui Brumm[c], Karen Nilsson[c], Volker Gerdts[d], Andrew Potter[d], Adam Polkinghorne[a,b], Kenneth Beagley[a,1], Peter Timms[a,b,*,1]

[a] Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Ave., Kelvin Grove, QLD 4059, Australia
[b] Faculty of Science, Health, Education & Engineering, University of the Sunshine Coast, Locked Bag 4, Maroochydore DC, QLD 4558, Australia
[c] Lone Pine Koala Sanctuary, Fig Tree Pocket, QLD 4519, Australia
[d] Vaccine and Infectious Disease Organizations, University of Saskatchewan, 120 Veterinary Road, Saskatoon, Saskatchewan, Canada

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ABSTRACT

Chlamydial infections are wide spread in koalas across their range and a solution to this debilitating disease has been sought for over a decade. Antibiotics are the currently accepted therapeutic measure, but are not an effective treatment due to the asymptomatic nature of some infections and a low efficacy rate. Thus, a vaccine would be an ideal way to address this infectious disease threat in the wild. Previous vaccine trials have used a three-dose regimen; however this is very difficult to apply in the field as it would require multiple capture events, which are stressful and invasive processes for the koala. In addition, it requires skilled koala handlers and a significant monetary investment. To overcome these challenges, in this study we utilized a polyphosphazine based poly I:C and a host defense peptide adjuvant combined with recombinant chlamydial major outer membrane protein (rMOMP) antigen to induce long lasting (54 weeks) cellular and humoral immunity in female koalas with a novel single immunizing dose. Immunized koalas produced a strong IgG response in plasma, as well as at mucosal sites. Moreover, they showed high levels of *C. pecorum* specific neutralizing antibodies in the plasma as well as vaginal and conjunctival secretions. Lastly, *Chlamydia*-specific lymphocyte proliferation responses were produced against both whole chlamydial elementary bodies and rMOMP protein, over the 12-month period. The results of this study suggest that a single dose rMOMP vaccine incorporating a poly I:C, host defense peptide and polyphosphazine adjuvant is able to stimulate both arms of the immune system in koalas, thereby providing an alternative to antibiotic treatment and/or a three-dose vaccine regime.

1. Introduction

Koala populations along the eastern coast of Australia face localized extinction due to anthropogenic pressures such as habitat destruction [1], motor vehicle trauma [2], bush fire, dog attacks [3] and disease [4]. Control measures targeting disease may reduce mortalities and as such could have the potential to stabilize declining koala populations [5]. Based on data collected from wild hospital admissions, *Chlamydia* is the most common cause of disease in koalas [6]. *Chlamydia* is an intracellular bacterium that causes disease, not only in koalas, but also in a wide range of wild and domestic animals and humans [7]. In koalas, *Chlamydia pecorum* is the most pathogenic species and is associated with

* Corresponding author at: Department of Microbiology, Faculty of Science, Health, Education & Engineering, University of the Sunshine Coast, Locked Bag 4, Maroochydore DC, QLD 4558, Australia. Tel.: +61 5456 5576/0431 709 745; fax: +61 7 5430 2889.
E-mail addresses: shahneazali.khan@student.qut.edu.au (S.A. Khan), cwaugh@usc.edu.au (C. Waugh), galit@koala.net (G. Rawlinson), jacqui@koala.net (J. Brumm), karen@koala.net (K. Nilsson), volker.gerdts@usask.ca (V. Gerdts), andrew.potter@usask.ca (A. Potter), apolking@usc.edu.au (A. Polkinghorne), k2.beagley@qut.edu.au (K. Beagley), ptimms@usc.edu.au (P. Timms).
[1] Joint last author.

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urogenital and ocular infections. Clinical signs include cystitis, sterility, infertility, conjunctivitis, kerato-conjunctivitis and rhinitis [8]. Antibiotics are the currently accepted therapy, however, they can have a deleterious effect on the animal's gastrointestinal microenvironment [9], as well as having quite low efficacy rates for chronic infections [10]. Further, many wild koalas have asymptomatic infections [11], challenging efforts to effectively treat and control disease in affected populations.

An effective vaccine would be an ideal disease management tool for koalas as in other host species infected with this bacterial pathogen. Across the broader chlamydial research field, the design of a successful vaccine has proven challenging, however, as researchers have had to consider both the selection of a suitable vaccine candidate capable of inducing immune-protection, and the development of an effective delivery system and adjuvant capable of boosting immune responses against the candidate antigens [12].

The most promising candidate for a chlamydial vaccine antigen is the chlamydial major outer membrane protein (MOMP), accounting for 60% of the chlamydial outer membrane [13]. A koala *C. pecorum* vaccine has been under development for the past four years using a recombinant MOMP (rMOMP)-based antigen [14,15]. The first koala vaccine trial demonstrated the induction of both cellular (>1 year) and humoral immunity (>35 weeks) in female koalas with a rMOMP-based vaccine combined with three different adjuvants, and identified the best adjuvant candidate as immunostimulating complex (ISC) [16]. The second trial elucidated the feasibility and safety of a *C. pecorum* specific rMOMP antigen combined with ISC as a vaccine in healthy as well as diseased female koalas [15]. The third trial identified the cross reactive nature of the monovalent rMOMP proteins in female koalas, which is useful as there are a significant number of genetically distinct *C. pecorum* strains circulating in wild populations [14].

While these results are promising, a limitation of the koala *Chlamydia* vaccine that is presently under development is that the adjuvant currently used requires a three (or two) dose regime [14]. This three dose regime would be logistically challenging to deliver to wild koalas while also potentially causing unnecessary stress to animals associated with repeated capture and handling. A single dose adjuvant that would deliver a similar level of immune recognition and response would be advantageous to plans to deploy this vaccine to koala populations across Australia.

This current study evaluated a novel one-dose vaccine in koalas. The adjuvant chosen consisted of three components, polyinosinic polycytidylic acid (poly I:C), a host defense peptide [17] and polyphosphazine (PCEP). Poly I:C is a TLR3 ligand that mimics viral double stranded RNA (dsRNA), the natural ligand of TLR3. From studies in other animals, poly I:C is known to induce a predominantly Th1 immune response [18] due to the strong induction of type 1 interferon production and proinflammatory cytokines. Host defense peptides (HDP) are cationic peptides that function as antimicrobials, have multiple immunostimulatory activities and are highly conserved across plants, insects and mammals [19]. Polyphosphazines such as PCEP are synthetic water-soluble and biodegradable polymers that have demonstrated strong adjuvant affects. PCEP induces the recruitment of myeloid and lymphoid cells to the injection site and draining lymph nodes [20], activates the NLRP3 inflammasome resulting in the production of pro-inflammatory cytokines such as IL-1β and IL-18 [21] and also induces potent mucosal IgA responses when delivered by multiple immunization routes [22].

Because it is widely believed that a protective chlamydial vaccine needs to elicit both a strong Th1 immune response at the infection site [18], as well as the production of neutralizing antibodies at the mucosal surfaces of the genital tract and eyes [23], we reasoned that this combination adjuvant should elicit such a balanced response. The present study therefore evaluated this three-component adjuvant, mixed with *C. pecorum* rMOMP and given as either a single or double dose immunization for induction of anti-chlamydial immunity in the koala.

2. Materials and methods

2.1. Production of rMOMP

Previously, Kollipara et al. [24] developed a typing scheme for *C. pecorum* strains infecting koalas. In our study, we used three koala *C. pecorum* genotypes (A, F and G) as they represent genotypes that are common in South-East Queensland (SEQ) koala populations. The purified *C. pecorum* MOMPs (A, F and G) were used as antigens in the current vaccine trial. Details for preparation of the recombinant MOMPs were as described by Kollipara et al. [14] but briefly, *Escherichia coli* (strains JM109; BL21 (DE3 pLysS)) were used for molecular cloning, protein expression and purification. The respective expression constructs were transformed into the *E. coli* and grown in Luria–Bertani broth with constant shaking at 37° C. The cell growth was assessed by measuring OD_{600}. The complete *omp*A genes for each *C. pecorum* strain were amplified using primers ompAXhol (5′-AAAAACTCGAGTTGCCTGTAGGGAACCC-3′) and OmpAKpnIn (5′-AAAAAGGTACCTTAGAATCTGCATTGAGCAG-3′). The PCR product was amplified to generate products consisting 5′-Xhol and 3′-KpnI restrictions and were ligated into a N-terminal polyhistidine (His) pRSET expression vector. His-tag expression constructs were then transformed into BL21 (DE3) pLysS bacterial cells and grown in Luria–Bertani broth medium. The cells (expressing rMOMP) were harvested by centrifugation and resuspended in lysis buffer I. Lyzed cells were incubated with TALON metal affinity resin at 4 °C for 1 h with gentle mixing. The resin then washed with buffer I and the protein was eluted. The recombinant protein yields were estimated by the bicinchoninic acid method.

2.2. Adjuvant

The combination adjuvant consists of polyphosphazine (PCEP), the host defense peptide HH2 (VQLRIRVAVIRA-NH_2) [17] and poly I:C (Vaccine and Infectious Disease Organization, Saskatchewan, Canada), which was combined with our rMOMP antigens. Each 500 μl dose of the vaccine was prepared using sterile PBS to contain approximately 50 μg of each rMOMP (A, F and G), with 250 μg each of PCEP, poly I:C and 500 μg of HH2.

2.3. Animals

Six healthy female koalas, which were seronegative for previous chlamydial infections, and aged 1–3 years, were used for this study and were housed at Lone Pine Koala Sanctuary, Brisbane, Queensland. There was no history of chlamydiosis in this facility for at least 10 years. All work was conducted under permissions from the Queensland University of Technology Animal Ethics Committee (permit # 0900000285).

2.4. Immunization schedule and sample collection

Koalas were randomly assigned into two cohorts. One group ($n = 3$) received a single dose of vaccine and the second group ($n = 3$) received two doses, with a one month interval between the doses. All the animals were immunized *via* the subcutaneous route by a registered veterinarian. Samples included 5 ml whole blood collected in EDTA blood collection tubes (Interpath Services), stored at 4 °C and processed within 24 h. The swabs were collected at the urogenital (UGT) and ocular sinuses (Aluminium rayon dry swabs; Copan) and were stored at −80 °C in 0.5 ml phosphate buffer solution (PBS) containing 1 mM phenylmethysulphonylfluride (PMSF).