Development of a chlamydial vaccine for koalas (*Phascolarctos cinereus*)

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Abstract

The koala (*Phascolarctos cinereus*) is an iconic arboreal marsupial and the only surviving member of the Family *Phascolarctidae.* Chlamydiosis in koalas causes significant morbidity and mortality and adds to the detrimental effects of anthropological changes such as deforestation, bush fire, motor vehicle trauma and dog attacks. Mathematical modelling suggests that by reducing the negative effects of chlamydiosis, koala population in decline could well be saved. *Chlamydia* is an obligate intracellular pathogen of both humans and animals and *C.pecorum* is the most common and serious species affecting koalas. Ocular infections in koalas cause kerato-conjunctivitis leading to blindness, whereas uro-genital infections cause thickening of the bladder wall, incontinence and fibrosis in the uterine tract. While antibiotics are the current leading curative measures, these are ineffective for severe chlamydiosis and can also affect the intestinal micro flora and the overall health of the animals. The asymptomatic nature of the chlamydial infection and the variable effects of the long term antibiotic treatment heighten the importance of developing a suitable anti-chlamydial vaccine.

The overall goal of developing an effective koala -*Chlamydia* vaccine requires a focus on exploring suitable vaccine antigens with immune stimulating adjuvants, to produce a long lasting cellular and humoral immune response. Our group has been developing a koala-*Chlamydia* vaccine over the past 6 years using the recombinant major outer membrane protein (MOMP). While the koala-*Chlamydia* vaccine looks promising, it still needs to address some critical aspects. The present study aimed to extend the previous work by (a) evaluating a simpler vaccine to administer, preferably single dose vaccination, utilising a novel adjuvant formulation, (b) understanding the detailed mechanism that underpin humoral immunity in both naturally infected and vaccinated koalas, (c) determining the therapeutic

and protective effects of the rMOMP vaccine strategy against the course of the infection in free-ranging koalas, and (d) understanding the role of the adjuvant on eliciting cellular and humoral responses in wild koalas.

In koala, the current vaccine regime utilized recombinant *C. pecorum* specific major outer membrane protein (MOMP) as the vaccine antigen. This protein represents 60% of the chlamydial membrane structure and consists of T and B cell epitopes. While MOMP is the subunit component of the chlamydial outer membrane protein, a suitable adjuvant formulation could further enhance its immunogenicity. Several immunisation studies have used ISC (Immune stimulating complex) adjuvant and this has provided the best immune protection to date. One disadvantage of ISC is it requires multiple immunisations to be efficient. This requirement for several immunisations is not ideal as it could cause additional stress to the koala through repeated capture and handling processes. Therefore, to overcome, the limitations of multiple vaccination schedules, we evaluated a combination adjuvant containing polyphosphazine based poly I: C and host defense peptides, which has previously been shown to be effective in other species after a single dose injection. In this current study, we demonstrated that this novel adjuvant elicited systemic and mucosal humoral immune responses against MOMP antigen.

Although Th1 immune response is critical in chlamydial infection, the role of antibodies has been described in a considerable number of research articles in mouse and guinea pig models, which supports the immune-protective role of antibodies. In this thesis, we have characterised the role of antibody mediated immune response in koalas with ongoing chlamydial infection either with vaccination or without vaccination. Particularly, our study in koalas has shown that antibodies induced through vaccination had neutralising ability and have unique epitopes

specificity apart from the natural infection. Interestingly, the vaccine induced epitopes are located in the conserved domains, suggesting their role in cross recognition against diversified MOMP genotypes. In broader perspective, we first examined the effect of chlamydial infection load against our prototype vaccine in free ranging koala. It has shown that the current vaccine was able to reduce the *Chlamydia* shedding in infected animals. The vaccine induced a significant immune response which might prevent new *C. pecorum* infection. This study strongly suggests the therapeutic effect of this vaccine through distinct epitopes specificity. Overall, the vaccine induced immunity prevents infection burden and increased the longevity of the animals.

The ideal vaccine against *Chlamydia* should elicit IFN-γ secreting CD4+ T cells and neutralizing antibodies at the infection site. In addition, the Th2 or antibody response prevents reinfection and a balance of these two mechanisms coordinates the key immune protective role. In the final study, we measured the cytokine gene expression of the PBMCs following vaccination with the two different adjuvants each combined with the same rMOMP protein antigen. Overall, both adjuvants produced a strong *Chlamydia*-specific cellular response in circulating PBMCs (peripheral blood mononuclear cells) as well as MOMP and functional antibodies. Whilst the immune responses were similar, there were differences between the adjuvants, particularly in relation to the specificity of the antibody responses. Together, these data suggest that a single dose vaccine (referred as Tri-adjuvant vaccine) regime appears as effective in triggering an anti-*Chlamydia* immune response in koalas.

Key words:

Chlamydia pecorum; adjuvant: single dose; MOMP; antibody; epitope; therapeutic; vaccine; immune response

List of publications associated with this thesis

Khan, S. A., Waugh, C., Rawlinson, G., Brumm, J., Nilsson, K., Gerdts, V., Potter, A., Polkinghorne, A., Beagley, K. and Timms, P. (2014). 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. Vaccine, 32(44), 5781-5786.

Khan, S.A., Polkinghorne, A., Waugh, C., Hanger, J., Loader, J., Beagley, K., and Timms, P. (2016). Humoral immune responses in koalas (*Phascolarctos cinereus*) either naturally infected with *Chlamydia pecorum* or following administration of a recombinant chlamydial major outer membrane protein vaccine. Vaccine. 34, 775-782.

Waugh, C, **Khan, S.A.,** Carver, S, Hanger, J., Loader, J., Polkinghorne, A., Beagley, K., and Timms, P. (2016). A Prototype Recombinant-Protein Based *Chlamydia pecorum* Vaccine Results in Reduced Chlamydial Burden and Less Clinical Disease in Free-Ranging Koalas (*Phascolarctos cinereus*). PLoS one; 11(1): e0146934.

Khan, S.A., Desclozeaux, M., Waugh, C., Hanger, J., Loader, J., Gerdts, V., Potter, A., Polkinghorne, A., Beagley, K., and Timms, P. (2016). Antibody and cytokine responses of koalas (*Phascolarctos cinereus*) vaccinated with recombinant chlamydial major outer membrane protein (MOMP) with two different adjuvants. PLoS One; 11(5): e0156094.

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List of abbreviations

MOMP	Major Outer Membrane Protein
NrdB	Ribonucleoside –diphosphate reductase subunit beta
EB	Elemenatry Body
RB	Reticulate body
CD	Conserved Domain
VD	Variable Domain
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
Ct	Threshold Cycle
Ig	Immunoglobulin
IFN	Interferon
TNF	Tumor necrosis factor
IL	Interleukin
Poly I: C	Polyinosinic polycytidylic acid
HDP	Host defense peptide
PCEP	Poly [di (sodiumcarboxylatoethylphenoxy) phosphazene]
MHC	Major Histocompatability Complex
PBMC	Peripheral Blood Mononuclear Cell
PMA	Para-Methoxyamphetamine

qrtPCR	Quantitative Real Time Polymearse Chain Reaction
UGT	Urogenital Tract
UV	Ultra Violet
CFSE	Carboxyfluorescein succinimidyl ester
ABTS	2, 2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)

Statement of original authorship

The research described in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institute. To the best of my knowledge and belief, the thesis contains no material previously published or written any person except where due reference is made.

QUT Verified Signature

Signature: Shahneaz Ali Khan

Date: 17/07/2016

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Chapter 1: Introduction

1.1 A description of the scientific problem investigated

Chlamydial infection is widespread in koalas and causes significant morbidity and mortality across the koala population in Australia [1]. *Chlamydia* infection directly threatens the survival of the koala and has been associated with ocular and reproductive illness. This infection is transmitted through sexual behaviour as well as vertical transmission from mother to joey during pap feeding. This disease can significantly reduce the reproductive health of the population and when combined with other anthropological pressures, can result in serious decline of the population. This infection is unfortunately the second most common reason for the admission of koalas to koala rehabilitation centres after car accidents. Disease modelling suggests that checking the chlamydial infection would be a significant management strategy that could help reverse the population decline [2]. While there are different grades of chlamydial infections, the chronic active chlamydiosis results in the shedding of the highest number of infectious particles, posing a serious impact for future transmission. However, the asymptomatic nature of the infection makes it difficult to deal with systemic antimicrobials. Therefore a vaccine would be an ideal option to control this infection.

The current vaccine utilising the recombinant major outer membrane protein (rMOMP) with multiple immunisations, elicited strong cellular and humoral immunity in koalas in several vaccine trials [3-6]. This multi-dose vaccine regime is problematic both logistically and financially to deploy in the wildlife settings. Therefore, to overcome the limitations of multiple vaccination schedules, a single dose vaccine with appropriate adjuvant would be an ideal option. Whilst MOMP is an immunogenic antigen accounting for 60% of the membrane structure [7] that constitutes B and T cell epitopes [8], it has been reported that there are some limitations of using rMOMP protein for vaccine development. Indeed, MOMP is (a) unable to elicit cross-serovar-protection and (b) requires adequate 3-D structure to restore the

required level of immunogenicity [9]. While MOMP is the leading subunit vaccine antigen and has shown good promise, the adjuvant component is equally important and further effort is still needed. Adjuvants are an important component of vaccine formulations and have major functions for enhancement of antigen uptake and presentation to the secondary lymphoid tissues. These immunomodulatory substances have multiple activities, including antigen delivery, recruitment of the specific immune cells to the site of immunisation and maturation of antigen presenting cells [10].

The ideal anti- *Chlamydia* vaccine should induce Th1 directed immune –protection with IFNγ secreting CD4+ T cells and neutralising antibodies at the infection site, given the evidence available from animal [11, 12] and human studies [13, 14]. The antibodies neutralise the infection at their entry as well as in FcR mediated phagocytosis and complement activation linked with T cell response [15-18]. There is still limited data available on antibody mediated immune response in koalas following either vaccination or infection. While the lack of a koala-*Chlamydia* infection model, the vaccine response in naturally infected animals could be an alternative option. Nevertheless, for the management of the wild animal's disease, it is significantly important to minimise the infection burden at population level and warrant the therapeutic and prophylactic vaccine as a practical solution. Animal model against chlamydial infection have shown the important role of Th1 directed cytokines, specially IFN-γ [13]. In the mouse model having strong IgA response without IFN-γ secretions fails to mount immune-protection. This pro-inflammatory cytokine inhibits the growth of *Chlamydia* through several ways [19, 20].

Taken together this several aspects of chlamydial immunity and vaccine research in koala, we aimed to investigate the following key aspects in this thesis.

1.2 The specific aims of the current study

The overall aim of the study described in this thesis is to contribute to the development of a *C. pecorum* vaccine for koalas. In our research we aimed:

1. Investigate the immune response of a novel Tri-adjuvant component polyphosphazine based poly I: C and host defense peptides combined with rMOMP *C. pecorum* antigen in koalas with single vaccination.
2. Characterise the antibody mediated immune response in koalas following either natural infection or vaccination.
3. Compare the immune response following MOMP vaccine and adjuvanted with either ISC or Tri-adjuvant.

1.3 Progress of research linking the scientific papers

This thesis consists of four papers, which have been published (4) in peer reviewed journals. The scientific work presented in these papers directly addresses the specific aims of this research project.

The first paper (Chapter 3) entitled "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" has been published in the "Vaccine" journal, Volume 32 on September 4, 2014. This paper describes that single dose rMOMP vaccine combined with a poly I:C, host defense peptide and polyphosphazine adjuvant is able to stimulate both cellular and humoral immune response in koalas. This is the first study in koalas that utilised these novel adjuvant components. The immune responses that we observed in koalas to rMOMP

incorporated with the Tri-adjuvant were similar to the previously described study. Although the Th1 immune response is critical in chlamydial infection, the role of antibodies has been described in a considerable number of published articles in laboratory animal models, though there was no detailed analysis of the humoral immune response to vaccine in koalas. This leads us to the next article describing the role of antibody against chlamydial infection and vaccination.

The second paper (Chapter 4) entitled "Humoral immune responses in koalas (*Phascolarctos cinereus*) either naturally infected with *Chlamydia pecorum* or following administration of a recombinant chlamydial major outer membrane protein vaccine" has been published in the "Vaccine" journal, Volume 34 on December 30, 2015. As part of the thesis work, we utilised a wild koala population to study (a) the immune response of naturally infected koala and (b) the immune response following vaccination. One of the important findings in this study was that a different and unique set of antibodies were induced by vaccination, compared to those from natural infections. All these epitopes were in the conserved domain and these would be conserved across the multiple ranges of MOMP genotypes. As koala chlamydial infection is wide spread and often asymptomatic in free ranging koalas, a therapeutic vaccine is significantly important. This leads us to the next paper describing the prophylactic and therapeutic effect of chlamydial vaccine.

The third paper entitled "A prototype recombinant-protein based *Chlamydia pecorum* vaccine results in reduced chlamydial burden and less clinical disease in free-ranging koalas (*Phascolarctos cinereus*)" has been published in the "PLoS One" journal, Volume 11, January 12, 2016. In the broader aspect of the larger koala vaccine project, this study confirms the effectiveness and protective immune response of *C. pecorum* positive koalas following vaccination. The vaccine induced very high antibody titers in koalas even for those

with current infections. Notably, the percent of neutralisation was significantly higher in the vaccinated group, compared to natural infections.

The final aspect of the thesis was to evaluate the adjuvant effects on the immune response of koalas and is depicted in the final article. This paper entitled "Antibody and cytokine responses of koalas (*Phascolarctos cinereus*) vaccinated with recombinant chlamydial major outer membrane protein (MOMP) with two different adjuvants" has recently been published in the "PLoS One" journal. In this study we first measured the T cell response through IFN-γ gene response in koalas. The animals showed comparatively similar immune response regardless of the two different adjuvant regimes. Both ISC and single dose Tri-adjuvant immunisation resulted in high antibody titers with good *in vitro* neutralisation levels.

Taken together, the results presented in chapters 3, 4, 5 and 6 clearly improve our understanding of chlamydial immune response in koalas in response to vaccine. These results will help us in targeting vaccine antigens along with appropriate adjuvant for future vaccine design in koalas against chlamydiosis.

1.4 Thesis outline

This thesis has been written according to Queensland University of Technology's (QUT) "PhD thesis by publications" guidelines. Chapter 2 describes the literature relevant to the field of this particular study. Then Chapter 3, 4, 5 and 6 describe published and submitted work in the peer reviewed journals as part of the thesis. Chapter 7 describes the significant findings of this research project along with a few outlines for future scientific work.

1.5 References

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Chapter 2: Review of literature

Chlamydia is a gram negative, coccoid intracellular bacterium that is pathogenic for humans and animals and causes a wide spectrum of diseases in the respiratory and uro-genital tracts as well as in the eyes. The Greek word *Chlamydia* means “cloak” or “a mantle” and describes the bacterial inclusions morphology around the host nucleus [1].

2.1 *Chlamydia*

Chlamydia is an obligate intracellular bacterium with a unique biphasic developmental cycle that exists inside a range of eukaryotic host cells [2]. This bacterium infects a wide range of animals as well as humans causing a wide spectrum of diseases in the eyes, respiratory and urogenital tracts [3, 4]. Currently, the genus *Chlamydia* has 11 recognised species: *C. abortus*, *C. avium*, *C.caviae*, *C. felis*, *C. gallinacea*, *C. muridarum*, *C. pecorum*, *C. psittaci*, *C. suis*, *C. pneumoniae* and *C. trachomatis*. The host range of these species varies. For example, *C. trachomatis* infects humans, swine and mice; *C. psittaci* infects mammals, birds, cats and guinea pigs; *C. pecorum* infects a wide range of animals including mammals (sheep, cattle, goats), marsupials (koalas) and swine; *C. pneumoniae* infects humans and other animals [5, 6].

2.2 Developmental cycle

Chlamydia has two morphologically and physiologically distinct developmental forms; the non-dividing, infectious elementary bodies (EB) and replicative, non-infectious reticulate bodies (RB) [7]. Another developmental phenotype characterised by an atypical aberrant body is associated with persistent infections of this pathogen. The infectious EB is small in size compared to RBs with highly condensed chlamydial DNA and histone-like proteins *hctA* and *hctB* [8]. While EBs are thought to be metabolically inactive, studies have shown that EBs exhibit a low-level of metabolic activity by utilising glucose-6 phosphate [9].

Initial attachment of *Chlamydia* EBs is thought to be a two stage process involving the electrostatic interactions of the bacteria with heparin sulphate proteoglycans [10], followed by high affinity irreversible binding which is mediated by an, as yet, unidentified secondary receptor [11]. However, mannose, mannose 6-phosphate as well as the estrogen receptor have all been suggested to play a role in *Chlamydia* entry in the host cell [12, 13]. Both infectious EBs and the host proteins function synergistically to promote the invasion process [14]. EBs have a unique cysteine-rich disulphide cross-linked outer membrane surface structure [15], which is osmotically stable and resistant to extracellular stresses [16].

Following entry, the EB is internalised into a membrane bound compartment termed an inclusion. The inclusion contains various biosynthetic precursors, amino acids and lipid substances from the host cells [17, 18]. The inclusion avoids fusion with lysosomes and subsequently moves to the peri-golgi region [19]. Additionally, the inclusion interacts with a number of cellular organelles including multivesicular bodies [20], lipid droplets [21], mitochondria [22] and lysosomes [23] to acquire the nutrients for replication and as well as for membrane stability. *Chlamydia* differentiates from the EB form into actively replicating RBs, predominantly by multiplying through binary fission within the inclusion [24]. Persistence can be induced during this stage through nutrient starvation, interferon-gamma stimulation, and induction of nitric oxide synthase or antibiotic treatment [25-27]. After completing the developmental cycle, RBs transform back to EBs through an asynchronous process with repackaging bacterial chromosomes, nutrients and type 3 secretions (T3S) [24, 28, 29]. Release of EBs can occur in two ways either via disruption of the host cell membrane through the lytic pathway or extrusion of the EBs from the host cell without disturbance of the cell membrane [30].

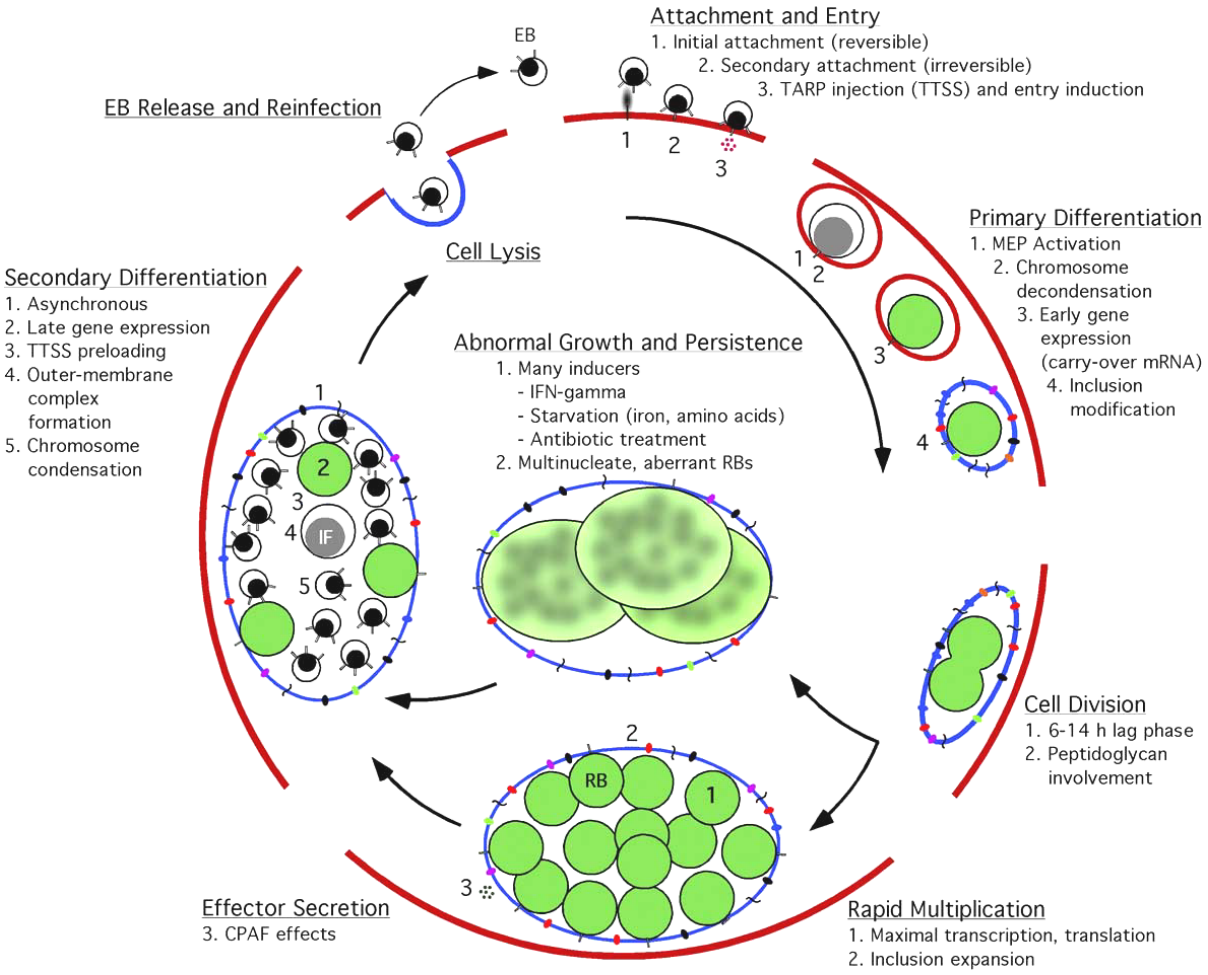


Fig 1. Diagrammatic presentation of the chlamydial developmental cycle in a typical host cell. It depicts the key events that occur inside the host cell as part of their developmental cycle. EB: Elementary body; RB: Reticulate body; CPAF: Chlamydial protease like activity factor; TARP: Translocated actin recruiting phospho-protein; MEP: Methylerythritol phosphate; TTSS: Type three secretion systems (Taken from: AbdelRahman and Belland, [31]).

2.3 *Chlamydia* Taxonomy

The taxonomy of *Chlamydia* is a debatable issue, which can be divided into two eras. According to the pre-1999 era, *Chlamydia* consisted only in one Family *Chlamydiaceae* and one genus *Chlamydia*. In this taxonomy, the genus *Chlamydia* contained four species: *Chlamydia trachomatis*, *Chlamydia psittaci*, *Chlamydia pneumoniae* and *Chlamydia pecorum*. Following the publication of Everett et al., in 1999, the genus *Chlamydia* was split into two genera namely *Chlamydia* and *Chlamydophila*, consisting of a total of nine species. *C.pneumoniae*, *C. psittaci*, *C. pecorum*, *C. abortus*, *C. felis* and *C. caviae* were under *Chlamydophila* and *C. trachomatis*, *C. suis* and *C. muridarum* were within the genus *Chlamydia*. In the twenty-first century, Stephens et al. rearranged and proposed a single genus, *Chlamydia*. In this nomenclature, nine species exist within the genus *Chlamydia* as such *C. trachomatis*, *C.pneumoniae*, *C. psittaci*, *C. pecorum*, *C. abortus*, *C. suis*, *C. felis*, *C. caviae* and *C. muridarum* [32]. Two new species have been recently added to the *Chlamydiaceae – C. avium* and *C. gallinacea*. *C. avium* has been isolated from a number of avian species including pigeons and parrots while *C. gallinacea* was isolated from chickens [33].

Table 1. Taxonomy of chlamydial organisms (Taken from Schautteet and Vanrompay, [34])

Chlamydial taxonomy before 1999			Chlamydial taxonomy since 1999 (Everett et al., 1999)				Chlamydial taxonomy used in the twenty-first century (Stephens et al., 2009)	
Order	Chlamydiales		Chlamydiales				Chlamydiales	
Family	*Chlamydiaceae*		*Chlamydiaceae, Simkaniaceae, Parachlamydiaceae, waddliaceae*				*Chlamydiaceae, Simkaniaceae, Parachlamydiaceae, waddliaceae*	
Genus	*Chlamydia*		*Chlamydia*		*Chlamydophila*		*Chlamydia*	
Species	*C. trachomatis*	Trachoma biovar	*C. trachomatis*	Trachoma biovar			*C. trachomatis*	Trachoma biovar
		LGV biovar		LGV biovar				LGV biovar
		Murine biovar	*C. muridarum*				*C. muridarum*	
		Porcine biovar	*C. suis*				*C. suis*	
	C. pneumoniae	Human biovar			*Cp. pneumoniae*	TWAR biovar	*C. pneumoniae*	TWAR biovar
		Koala biovar				Koala biovar		Koala biovar
		Equine biovar				Equine biovar		Equine biovar
	C. psittaci	Avian subtype			*Cp. psittaci*		*C. psittaci*	
		Abortion subtype			*Cp. abortus*		*C. abortus*	
		Feline subtype			*Cp. felis*		*C. felis*	
		Guinea-pig subtype			*Cp. caviae*		*C. caviae*	
	C. pecorum				*Cp. pecorum*		*C. pecorum*	

2.4 Diseases caused by *Chlamydia*

Chlamydia causes a wide spectrum of diseases in different hosts [4]. Trachoma caused by *C. trachomatis* was one of the earliest ocular disease in both male and females, recorded by Hippocrates as far back as 420 B.C. Diseases associated with *C. trachomatis* can be separated by serovars based on the epitope recognition of the 4 variable regions, the MOMP protein and nucleic acid sequence of *ompA*. The genital serovars (D-K) of *C. trachomatis* are the leading pathogens for the sexually transmitted diseases (STDs) in humans, with over 100 million cases reported annually worldwide [35]. The ocular strains of the *C. trachomatis* (serovars A-D) are associated with blindness in developing countries [36]. The genital form of infection in women, causes cervicitis, urethritis, endometritis, pelvic inflammatory disease (PID), infertility with tubal occlusion [37, 38]. In men, it has been associated with urethritis, epididymitis and prostatitis [39-41]. Whilst, the majority of the *C. trachomatis* infections are asymptomatic, severe complications resulting from chronic infection have been seen in both men and women. The Lymphogranuloma serovars (L1, L2 and 3) are invasive in nature and predominantly restricted to the mucosal epithelium and submucosa of the affected individual, thereby causing lymphadenopathies, proctitis and ulcerative lesions in the intestinal mucosa [42].

The mouse species of *C. muridarum* was initially isolated from the respiratory tracts of mice but the pathogen can be transmitted through an oral route. It infects both the respiratory and genital tract in mice, similar to *C. trachomatis* genital infection in humans [43], paving the way for utilising the mouse and *C. muridarum* as laboratory animal infection model for the study of human respiratory and sexually transmitted chlamydial infections [44].

C. suis, has only been isolated from pigs, in which it may be endemic. This species of *Chlamydia* is associated with conjunctivitis, pneumonia and enteritis in both farmed and wild pig populations [45]. While the zoonotic potential of *C. suis* has not been exclusively examined, there is some indication that this pathogen can cause conjunctivitis in pig abattoir employees [46].

Psittacosis is a systemic infection caused by *C.psittaci* in domesticated and feral birds including chickens, pigeons and turkeys. This pathogen causes systemic infection in birds and the symptoms include conjunctivitis, difficulty breathing and watery droppings [47]. All avian species are likely to be the natural hosts and the infection is transmissible to humans with nonspecific clinical symptoms [48]. The first documented human psittacosis or popularly "parrot fever" was described in the early 1900s [49].

C. pneumoniae causes bronchitis and pneumonia in humans [50], but it has also been associated with a number of other diseases such as coronary heart diseases [51], Alzheimer's [52], reactive arthritis [53] and asthma [54]. This pathogen also infects a wide range of animals including koalas [55], horses, frogs and turtles [56]. Unlike other human species, *C. pneumoniae* exhibits a highly conserved *ompA* sequence, with little difference between human and animals strains [57]. Though animal to human transmission has been suggested on at least in two occasions [58], no evidence for human to animal transmission has been reported.

C. felis is a natural pathogen of domestic cats and causes hyperaemia of nictitating membrane, blepharospasm, ocular discomfort and conjunctivitis [59]. It causes conjunctivitis in humans and the prevalence of human infection is 1.7% [60].

C. abortus causes abortion and mastitis in cattle and ovine enzootic abortion in lambs [61]. The pathogen was first identified in sheep with abortions and thus causes serious economic problems worldwide. This pathogen has been documented in other livestock species as well as rodents [62].

C. caviae causes infections in its natural host, guinea pigs, and is mainly associated with guinea pig inclusion conjunctivitis. This pathogen is highly host-specific and the reproductive infection in guinea pig has been used as an experimental animal model to evaluate the pathogenesis of *C. trachomatis* genital infection [63].

C. pecorum was first isolated from a calf with encephalomyelitis [64] and later on has been found in a number of livestock and marsupial hosts. This pathogen causes a wide spectrum of diseases such as conjunctivitis, pneumonia, poly-arthritis, intestinal infection, encephalomyelitis in a broad range of animals including koalas, bandicoots, ruminants, pigs and horses [3, 65, 66]. *C. pecorum* is well studied in koala, where it has showed genetic diversity due to cross-species transmission within the wild population [67].

Two new species have been added to the *Chlamydiaceae*: *C. avium* and *C. gallinacea*. These two species were isolated from avian species including chickens, pigeons and parrots. These pathogens cause respiratory illness, enteritis and hepatic enlargement in their host animals [33].

Table 2. Epidemiology of chlamydiosis at the interface of the human-animal niche (Taken from Rodolakis and Yousef , [68])

-[b] not reported

***Chlamydia* species**	**Host**		**Pathology in animals**		**Route for human transmission**	**Pathology in humans**	
	Principal host	**Occasional host**	**Clinical signs**	**Severe disease**		**Usual disease**	**Severe disease**
C. psittaci	Bird	Dog, horse, pig	Hyperthermia, anorexia, lethargy, diarrhoea	Conjunctivitis, pneumonia, pericarditis, death	Inhalation	Influenza like illness	Endocarditis Encephalitis, pneumonia, death
C. abortus	Sheep, goat, cattle	Pig, deer, horse	Abortion, stillbirth, epididymitis	Metritis	Inhalation	Influenza like illness	Pneumonia, abortion, renal failure, respiratory distress, death
C. felis	Cat	-[b]	Conjunctivitis	Pneumonia, chronic sulphingitis	Contact	Conjunctivitis	Endocarditis, severe liver break down
C. caviae	Guineapig	-	Genital tract infection	-	Contact	Conjunctivitis	-
C. pneumoniae	Human, koala, horse	Reptiles, amphibians	Respiratory disease	-	Inhalation	Pneumonia, bronchitis, asthma	Atherosclerosis
C. pecorum	Ruminant, swine, koala	Wild animals	Intestinal infection, conjunctivitis, urinary tract infection	Encephalomyelitis, pneumonia, arthritis	Contact	-	-

2.5 Koala *Chlamydia* taxonomy

Initially, chlamydial species in koalas were known as *C. psittaci* based on cell culture observations. Restriction enzyme and gene probe analysis subsequently revealed two distinct types of *C. psittaci*, isolated from the conjunctiva and urogenital tract and rectum in koalas [69]. However, based on nucleotide sequencing of the *ompA* gene, these were further reclassified into *C. pneumoniae* and *C. pecorum* respectively [70-72]. In 1999, a revised taxonomic division temporarily moved these two species, with seven other known species, into a new genus *Chlamydophila* [73]. This amendment was not widely established and revisions merged all nine species into a single genus, *Chlamydia* [32]. The species infecting koalas are now referred to as *Chlamydia pecorum* and *Chlamydia pneumoniae*.

2.6 Epidemiology of koala *Chlamydia*

Chlamydia infection is widespread in wild koalas across their habitat in the northern region of Australia, including Queensland and New South Wales [74]. A variety of approaches have been used to measure the infections in koalas over the last 20 years. While serological assays were used in the early studies, recently, molecular techniques such as specific PCR methods have provided accurate data on the prevalence of chlamydiosis in koalas [75]. White et al. [76] conducted a molecular study to understand the epidemiology of chlamydiosis in koalas. This study demonstrated a high prevalence of *Chlamydia* in both male and female koalas at both anatomical sites (ocular and urogenital). Later on, Jackson et al. [77] utilised molecular methods to identify the epizootiology of the two chlamydial species within two different geographic wild populations. This study demonstrated infections caused by the *C. pneumoniae* were usually of lower intensity in comparison with *C. pecorum* infections. The study further revealed the vertical transmission pattern of the chlamydial infections within wild koala population. The increasing use of species-specific PCR and sequencing data has

revealed certain koala populations in the North-Eastern region of Australia have infection levels as high as 72-100% [75]. Chlamydiosis was unfortunately also the second most frequent reason for koala admission to a koala hospital or a koala care centre in New South Wales over a 30 year period [78].

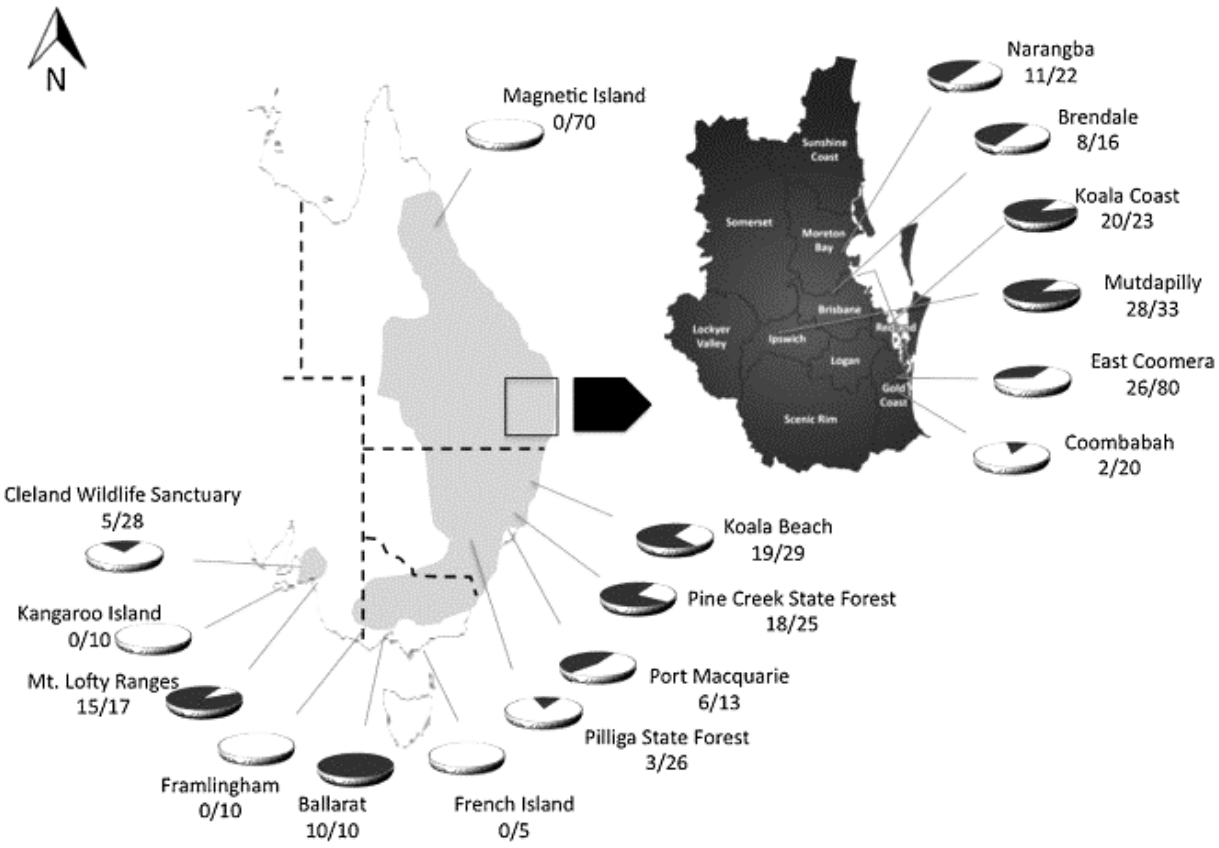


Fig 2. The map illustrating the prevalence of *Chlamydia pecorum* across the eastern half of Australia indicating the known geographic range of koala; Grey shading representing the koalas distribution (Taken from Polkinghorne et al. [75]).

2.7 Types of koala-*Chlamydia* infection

Koalas exhibit four types of clinical presentation of disease syndrome associated with chlamydial infection. In ocular infections, the inflammation is confined in the mucosal epithelium and is characterised by serous ocular discharge, blepharospasm and purulent discharge with conjunctivitis, fibrosis and blindness. In severe and chronic cases, corneal opacity can occur. Urinary tract infection manifests by brown urinary discharge, involuntary urination resulting in wet bottom or dirty tail around the rump region, popularly known as wet bottom syndrome [79]. Rhinitis with complex respiratory disorders has been seen in respiratory illness. The reproductive symptoms are the significant devastating aspect of this disease, affecting both male and female koalas. In male koalas, infections are typically associated with conjunctivitis and genital infection can cause orchitis, epididymitis and prostatis [80]. Acute reproductive tract disease associated with severe inflammation in the mucosal epithelium reversibly (infertility) or irreversibly (sterility), in female koala, results in reproductive loss [81].

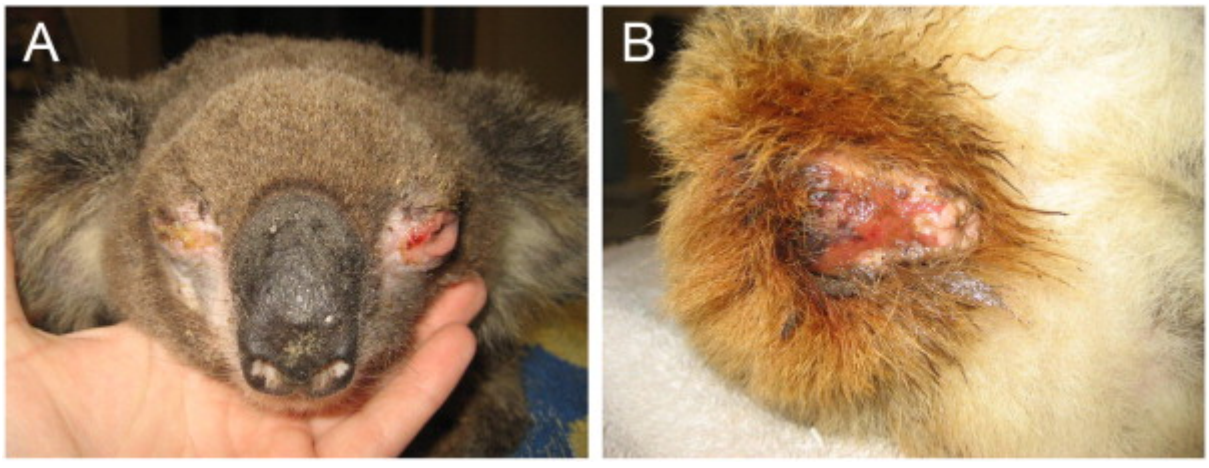


Fig 3. Clinical signs of chlamydiosis in koalas (a) the classical bilateral kerato-conjunctivitis and (b) wet bottom or dirty tail (Taken from Polkinghorne et al.[75])

2.8 Pathological lesions

The majority of the histopathological findings are confined to the lower genital tract of the reproductive tract in female genital chlamydiosis. In male koalas, mild inflammation has been seen within sertoli cells and interstitial spaces with intact seminiferous and epididymal tubules [80]. The gross pathological changes are characterised by marked and irregular thickening of the bladder wall; reduced diameter of the lumen has been noted [82]. Chronic disease is associated with cystic changes in the oviduct and ovarian bursae and in some cases thickening of the uterine wall. Prostatic abscess formation is the prominent pathological finding for male koalas [83, 84]. Ocular infections result in inflammation, characterised by conjuctival hyperplasia and progressing to fibrosis. In severe cases, corneal opacity develops and progresses to the rupture and collapse of the globe [75].

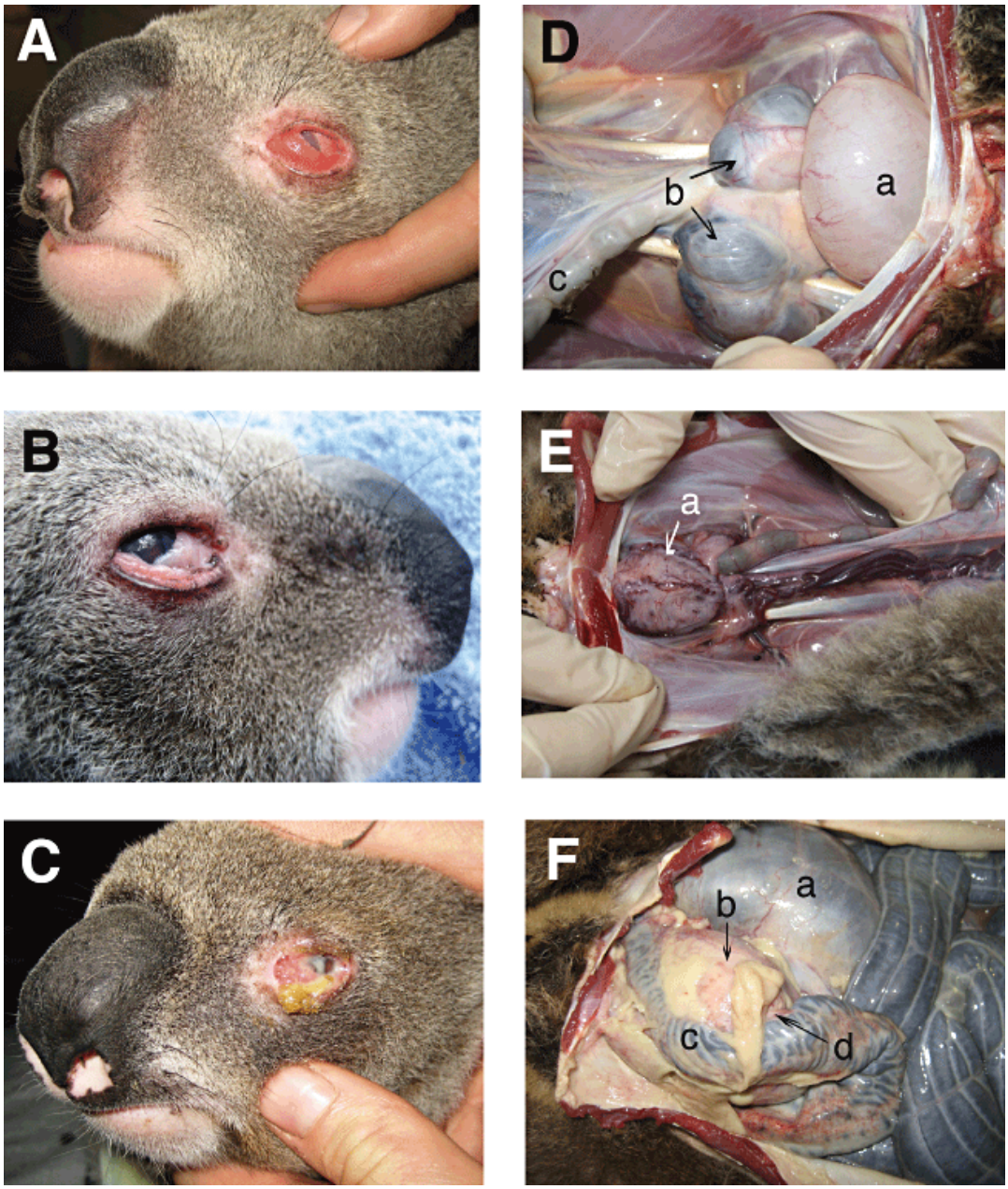


Fig 4. Clinico- pathological manifestation of chlamydial infection at ocular (A-C) and urogenital (D-F) sites. A) Acute kerato-conjunctivities with inflamed conjunctiva with minimal hyperplasia; B) Chronic-inactive kerato-conjunctivitis with extensive hyperplasia; C) Chronic active kerato-conjunctivitis with marked hyperplasia and exudation; D) Chronic , inactive urogenital infection with ovarian bursal cysts; E) chronic cystitis and F) severe urogenital tract pathology with ruptured uterine abscess (a. bursal cyst; b. uterine abscess; c. caecum; d. suppuratives exudates) [85].

2.9 Immunity against *Chlamydia*

The principal requirement for the immunological response of chlamydial pathogenesis is the T cell response in host defense against chlamydial infection. CD4+ T cells as well as CD8+ T cells are found at the infection site against *C. trachomatis* infection in human and mouse models [86, 87]. Antigen presenting cells (APC) are able to phagocytose chlamydial EBs, degrade chlamydial components into peptides and present them in conjunction with MHC class II to the CD4+ T cells. These T cell subsets can recognise *C. trachomatis* antigens, such as MOMP, polymorphic outer membrane protein as well as heat shock protein 60 (hsp60) [88, 89]. However, natural immunity against *C. muridarum* genital infection is mediated through CD4+ T cells, Th1 cytokines and antibodies. Whilst Th1 mediated response has been associated with protection, a Th2 mediated response is linked to immuno-pathology. The Th1 response is characterised by the production of IL-12 and IFN-γ and Th1- associated antibodies like IgG2a and IgG3 in the mouse model [90]. It is also evident that all chlamydial infection does not exclusively result in pathological damage and disease sequelae. Nonetheless, microbiological as well as host factors play a critical role in chlamydial disease pathogenesis. Indeed, the polymorphic characteristics of TNF-α and IL-10 promoter genes are able to affect the immune response against *C. trachomatis* human infection [91, 92].

2.9.1 Innate immune response

The first line of defense against chlamydial infection is polymorphonuclear cell activation through the Toll like receptor (TLR) pathway. This host innate immune response elicits chemokines and cytokine production in animal models within 24 h of infection [93, 94]. This early stage of the immune response is critical in controlling the primary infection until the initiation of an adaptive response [95]. In addition, natural killer (NK) cells contribute to enhance the innate immune response through the production of IFN-γ against *C. muridarum*

genital tract infection [96]. Human NK cells are able to lyse the *C. trachomatis* infected cells at the genital mucosa site [97]. Whilst the genital epithelial cells in response to *C.trachomatis* infection produce IL-18, the dendritic cells (DCs) generate IL-12 [98]. Both these cytokines are able to induce IFN-γ production from NK cells. The production of IFN-γ further initiates the differentiation of Th1 cells as well as increasing the degradation of tryptophan, in combination with TNF-α, IL-1 and lipopolysaccharide (LPS) [99].

2.9.2 Role of antibody

Different animal studies have addressed the role antibodies in immune protection [94]. In the guinea pig model, a strong antibody response was demonstrated following genital chlamydial infection [100]. Furthermore, this study confirmed that the role of antibody is essential for both controlling and eliminating the infection. In addition, the guinea pig model revealed that the animals are unable to resolve the primary infection, even though cellular immunity is activated [94, 101]. In murine models, B cells promote the protective T cell responses in the genital mucosa following chlamydial infection [102]. There is reported evidence that the anti-chlamydial antibodies are able to covert Th1 activation of T cells through FcR mediation [103]. Mucosal immunity is very crucial as chlamydial infection localizes in the reproductive and conjunctival mucosal layers and its replication, excretion and subsequent dissemination of the infection occurs through these natural orifices [104]. IgG is the most powerful immunoglobulin subclass that provides antibody mediated immunity at genital mucosa, as reported from several animal models [101,104, 105].

2.9.3 Cellular immunity

Several animal studies clearly indicate that a cell mediated immune response is required for both resolution of infection and immune protection [94,106, 107]. B cell deficient mice were

able to display immune protection against reinfection, which indicates the important role of T cells in eliciting protective immunity [108]. The Th1 cellular responses, specifically CD4+ T cells, are responsible for resolving genital chlamydial infection. The protective immune response is mediated through the induction of IFN-γ, either by CD4+ or CD8+ T cells [109, 110]. DC cells play a central role for T cell priming and subsequent induction of chlamydial immunity. This cellular response might be affected by hormonal changes as such oestradiol and progesterone, at the time of infection [111]. In addition to IFN-γ, IL-17A has been suggested to be a critical cytokine for protection against chlamydial infection, both for protection as well as in disease pathology [112, 113]. Nonetheless, an elevated IL-17A has been demonstrated against chlamydiosis in the mouse model [114].

2.10 Vaccine-induced immune response

Chlamydial vaccine research in animal models indicates the importance of both cellular and humoral immune responses for effective vaccine production [104]. As an intracellular pathogen, chlamydial infection induces antigen specific cell mediated immune response, mediated by T-helper cell types 1 (Th1) [115]. Indeed, the antibody action is significantly dependent upon T cell mediated adaptive changes at the infection site. However, *Chlamydia* specific antibodies play a critical role in protective immunity [116, 117] through neutralisation [118]. While the surface-acting antigen predominantly neutralizes through antigen-specific antibodies [119], a recent study suggests the role of hidden or unexposed epitopes in the neutralisation process through FcRn mediation [116, 117]. Similarly, antibody-deficient guinea pigs were unable to clear the infection, although the cellular immunity was intact [120]. Nevertheless, these animals were even more susceptible to reinfection following antibiotic treatment. Nonetheless, a recent study demonstrated that a peptide-based vaccine is feasible against chlamydial infection. This study was targeting

specific epitope recognition site at the rMOMP to enhance the production of neutralising antibodies [121].

2.11 Overview of chlamydial vaccine development

Current challenges in the vaccine design and development process attributes the selection of immune dominant antigen capable to elicit immune-protection [122]. In chlamydial vaccine development, a range of antigens has been evaluated in different animal models for their ability to induce an immune response.

2.11.1 Whole organism vaccine/1st generation vaccine

The initial animal and human vaccine studies against chlamydial infection were primarily based on either inactivated or live whole organisms [123]. The two animal pathogens *C. abortus* and *C. felis* have been successfully incorporated as whole organism killed or attenuated vaccines previously. These two vaccines are commercially available in the market and have been utilised against ovine enzootic abortion (OEA) and feline chlamydiosis [124, 125]. Unfortunately, similar vaccine approaches have proved unsuccessful against human chlamydiosis. In humans, initial partial immunity was developed against conjunctival infection but subsequent re-infection exacerbated disease pathology for certain individuals and this lead to the termination of this vaccine approach [126]. Recent study showed that there was no convincing evidence that vaccination led to more severe disease in humans [127].

2.11.2 Subunit /2nd generation vaccine

To overcome the adverse effects of the whole organism vaccine formulations, development of subunit vaccines has become the dominant strategy, with some good success. This

approach to vaccine development has the advantage of being safer and able to screen out the undesirable antigenic response related to adverse immune pathology [128]. MOMP is the leading subunit vaccine candidate in early vaccine trials and has been significantly used throughout the years in various animal models (Table 3 and Table 4)[111]. MOMP was used either as extracted outer membrane complex, as purified native or recombinant protein in the form of peptide, or as plasmid and as DNA (Table 4).

2.12 The vaccine antigen

The most promising antigen for developing an efficient chlamydial vaccine is MOMP (major outer membrane protein) (nMOMP, rMOMP, peptide or DNA MOMP). MOMP accounts for 60% of *Chlamydia* surface proteins and has a molecular mass of ~ 40 kDa [129]. DNA sequencing analyses revealed five constant (CD) and four variable (VD) domains across the full length of the MOMP protein [130-132]. This variable nature of sequence helps to evade the immune system of the host. Diversified VDs play a key immunodominant role in all chlamydial strains, except *C. pneumoniae* [133, 134]. Vaccination with recombinant MOMP antigens has shown promising results (see summary in table 3). Immunisation of mice and monkeys with the native form of MOMP (nMOMP) produced significant levels of immune-protection against genital and ocular challenge infections [135]. Murine and monkey models have shown strong protection against respiratory and intrabursal challenges by utilising the native form of MOMP (nMOMP) with oligodeoxynucleotide (ODN) as an adjuvant [136]. MOMP based-DNA vaccination had shown protection against an experimental respiratory infection in turkeys and budgerigars [137, 138].

Table 3. MOMP based vaccines in animal model against chlamydial infection

Vaccine antigen	**Adjuvant/delivery system**	**Model**	**Route of administration**	**Genus**	**No. of immunisations**	**Immune status/protection**	**Reference**
nMOMP, rMOMP	CpG-Montanide ISA 720	Mice	I/m and s.c	*Chlamydia trachomatis mouse pneumonitis*	Two times	Increased *Chlamydia* specific antibodies	[139]
DNA MOMP	Cathionic polymers	Turkey	I/m and I/n	*Chlamydia psittaci*	One time	Moderate immunoglobulin's in plasma	[137]
Plasmid DNA MOMP	GM-CSF, LTA, B and CpG	Pig	Intravagi nal	*Chlamydia trachomatis E*	One time	Reduced macroscopic pathological lesions Reduced vaginal shedding of *Chlamydia*	[140]
Bacteriophage-MOMP	Commercial vaccine strain 1B (Enzovax)	Mice	I/m	*Chlamydia abortus*	Two times	Moderate antibody response Production of high level of IFN-γ and IL-2	[141]
Ct-F-MOMP	CpG-2395 and Montanide ISA 720 VG	Monkey	I/m and s.c	*Chlamydia trachomatis F*	Two times	High level of *Chlamydia* specific IgG and IgA in plasma and natural secretions Detected cell mediated immune response in the peripheral blood mononuclear cells	[142]
nMOMP	CpG-CTB	Mice	I/m and s.c	*Chlamydia trachomatis*	Two times	High level of *Chlamydia* specific IgG in serum Significant T cell mediated *Chlamydia* specific immune responses	[143]
MOMP	Vibrio cholera ghost (rVCG)-CTA2B – nontoxic derivative of cholera toxin	Mice	I/m; I/v and transcuta neous	*Chlamydia trachomatis D and Chlamydia muridarum*	Two times	Trends of Th1 biased immune response Increased specific mucosal and systemic antibody Cross protection against heterologous chlamydial serovars	[144]
Multi epitopes based peptide MOMP	Human papilloma virus like particles (VLP)	Mice	I/m	*Chlamydia trachomatis*	Four times	Th1 biased immune response Upregulation of cytotoxic T lymphocyte activity	[145]
rMOMP	CpG-Montanide	Mice	Intravagi nal and I/m	*Chlamydia muridarum*	Three times	Significant protection against live challenges	[146]
rMOMP	Cationic liposomes (CAF01)	Mice	s.c	*Chlamydia muridarum*	Two times	Significant protection against live challenges	[147]
nMOMP	CpG-ODN-2395 and Montanide ISA 720	Monkey	I/m and s.c	*Chlamydia trachomatis*	Two times	High serum IgA and IgG at mucosal and systemic sites	[148]
nMOMP	CpG-1826 and Montanide ISA 720	Mice	I/m and s.c	*Chlamydia trachomatis mouse pneumonitis*	Two times	Increased secretion of antibodies in plasma Increase lymphopoliferative response of T cells	[149]
rMOMP	CpG-10109 and Cholera toxin	Guinea pig	Intranasa l	*Chlamydia trachomatis*	Three times	MOMP specific IgA and IgG in the vaginal wash	[150]

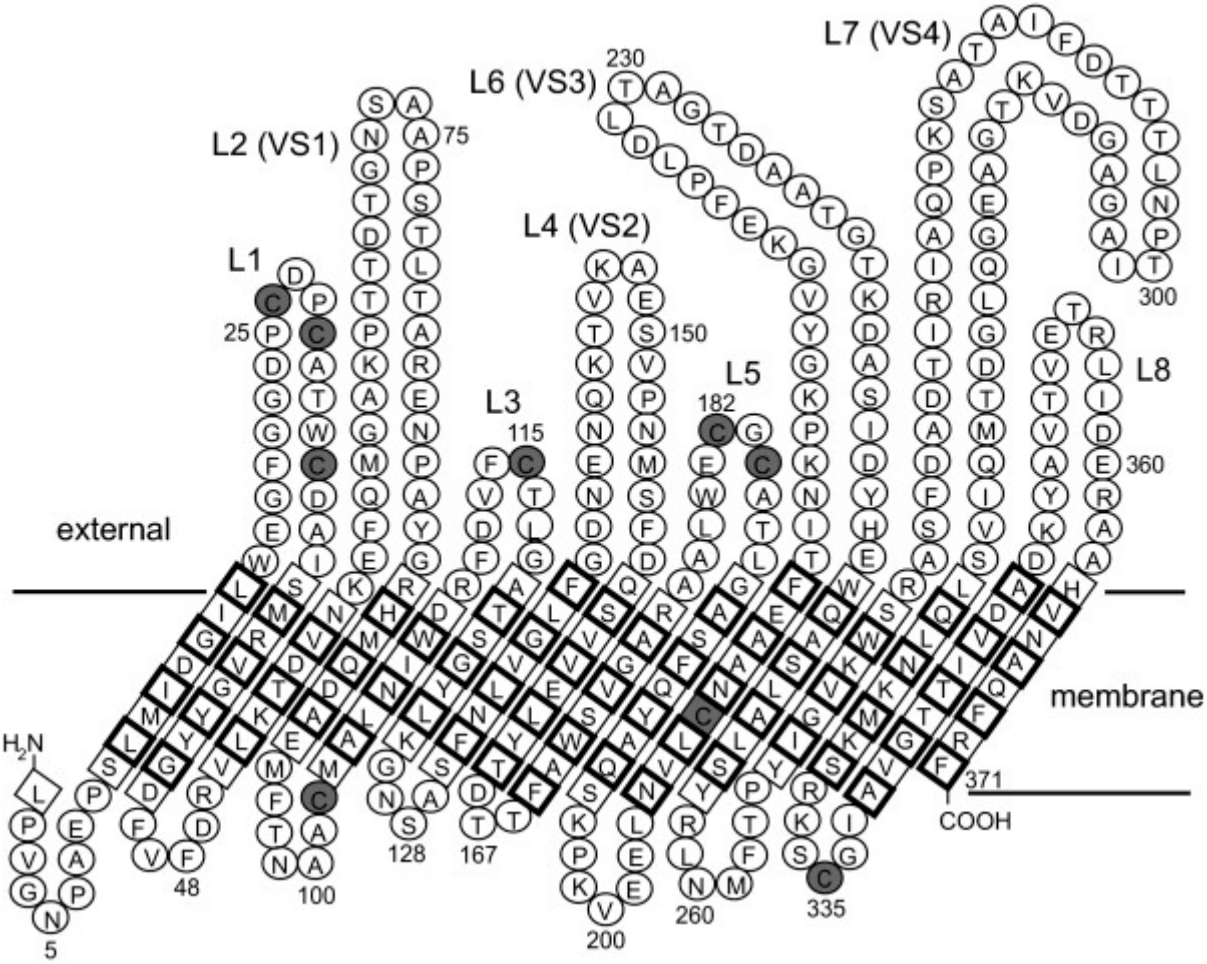


Fig 5. *C. trachomatis* MOMP diagrammatic structure. The residues in the trans-membrane strands are boxed with bold border and the side chains facing the lipid bi-layer are shown (both NH2 and COOH terminal ends of the protein are internal). External loops (L1-L8) with Variable domains 1, 2, 3 and 4 (VS1-4) are labelled with cysteine residues that are shaded. (Figure taken from Findlay et al.[151])

While MOMP has received the most attention, the construction of complete genome libraries and expression of specific predicted proteins have identified a range of immunogenic proteins as vaccine antigens [152, 153]. A few of them are described below.

Sequence analysis has identified 9 and 21 surface exposed polymorphic membrane proteins (Pmps) in *C. trachomatis* [154] and *C. pneumoniae* [155] respectively. Pmps play critical roles in chlamydial biology and virulence [156, 157], as they represent 13.6% and 17.5% of the coding capacity of these two genomes, It is suggested that these diversified proteins play vital roles in escaping from host immune surveillance [158, 159]. The *pmp* genes are predicted to encode membrane proteins to be localized in the outer membrane, due to the tryptophan residues in the C-terminal half and a C-terminal phenylalanine residue [160-163]. Genomics study revealed, the Pmp proteins are unique to the *Chlamydiales* family, and contain multiple GGAI and FXXN repeated motifs, associated with host cells adhesion [154, 164]. Pmps stimulate CD4+ T cells and pro-inflammatory cytokine production in murine chlamydial infection with *C. pneumoniae* [165-167].

Moreover, several Pmp proteins, Chlamydial protease like activity factor (CPAF), Plasmid encoded Pgp3 protein, Outer membrane complex protein (OmcB), Porin B protein (PorB), and Inclusion membrane protein (Incs) have also become leading vaccine candidates for *Chlamydia*. OmcB, rich in cysteine [168-170], is the second most abundant outer membrane protein complex, encoded by a bicistronic operon [171, 172] . This protein is highly conserved among *Chlamydia* [169], and has been involved to the conversion process of RBs to EBs during chlamydial infection [173], inducing humoral immune response in humans and animals [174, 175]. Cationic adjuvant formulation (CAF01) consisting of DDA as a delivery vehicle and synthetic mycobacterial cordfactor as immunomodulatory.

(Dimethyldioctadecylammonium (DDA) bromide and a, a'-trehalose 6, 6'-dibehenate (TDB), facilitates a Th1 biased immune response. Furthermore the adjuvant is stated to triggers both arms of the immune system and had shown promising results in experimental malaria, tuberculosis and chlamydial vaccines [176].

CPAF was the first established virulence factor secreted by *Chlamydia* in the cytosol [177], degrading host transcription factors RXF5 and upstream stimulation factor 1 (USF1), suppressing interferon-γ mediated expression of MHC I and II in infected cells, that may aid in chlamydial evasion of host immune system [178-180]. In addition, CPAF can cleave the cytoskeleton, facilitating expansion and growth of the inclusion [181]. During the developmental cycle, all *Chlamydia* multiply within a host cell derived vacuole termed an inclusion, which has insertions of specific bacterial proteins (Inc proteins). The major function of the Inc proteins is to interact with host cell components and elicit cell mediated immune response following chlamydial infection [182].

Table 4. Summary of *Chlamydia* vaccine research with major antigen-adjuvant approaches. i.m: intracmuscular; i.n: intranasal; s.c: subcutaneous; i.v: intravenous [181]

Vaccine	**Antigen and adjuvant**	**Immunization route**	**Model/*Chlamydia* infection route**	**Immune response**	**Reference (s)**
Intact *Chlamydia*	Plasmid-deficient *Chlamydia* (CM972, CM3.1)		Mouse/i.v	Elevated IgG2a (Th1), low levels of IgG1(Th2)	[184]
	Plasmid deficient *Chlamydia* (L2)		Mouse/i.v	Elevated IgG2a, low IgG1, no IgA (mucosal)	[185]
Purified subunits	MOMP plus cholera toxin subunit B conjugated to CpG	i.m + s.c	Mouse/i.n	Elevated IgG2a and IgG3 (Th1), lower IgG1 level, elevated IFNγ (Th1)	[143]
	MOMP-ISCOM	i.n or i.m	Mouse/i.n	i.m route induced highest IFNγ and IL4 (Th2) levels	[186]
	MOMP plus Freund's adjuvant	i.m + s.c	Mouse/i.v	Vortexted MOMP elicited higher IgG2a than IgG1 Sonicated MOMP elicited higher IgG1 than IgG2a	[187]
	MOMP plus IC31	i.m + s.c	Mouse/i.n	Higher IgG1 than IgG2a	[188]
	MOMP plus CpG/Montanide	i.m + s.c	*Rhesus macaque*	Elevated IgG, IgA, IFNγ and TNFα	[189]
Recombinants protein	rMOMP plus cholera toxin/CpG or CTA1	s.1 or. T.c or i.n	Mouse/i.n	Elevated IFNγ and TNFα	[190]
	rMOMP plus CpG/Montanide	i.m + s.c	Mouse/i.n	Vaccination protected against fibrotic scarring in lungs	[191]
	rCPAF plus Il12	i.n	Mouse/i.v	Increased IFNγ and minimal IL4	[192]
	rCPAF plus CpG	i.n	Mouse/i.v	Vaccination significantly prevented infertility	[193]
	rCTh1 plus CAF01	s.c	Mouse/i.v	T cell production of TNFα, IFNγ and Il2	[194]
	rGlgP plus CpG	i.m	Mouse/i.v	Th1 dominat T cell response	[195]
	rMIP	i.m	Mouse/i.v	Elevated IFNγ and no IL4	[196]
	rCT043	i.m	Mouse/i.n	Reduces bacterial load	[197]
	rCT823 plus ISCOM and CT144 plus ISCOM	s.c	Mouse/i.v	Elevated IFNγ, TNFα and IL2	[198]
	rPmpG plus GNE and SctC plus GNE	s.c	Pig/i.v	PmpG protected better than SctC PmpG vaccination did not elicit antibody production SctC vaccination elicited high antibody titers	[199]
DNA vaccine	DNA MOMP	i.m	Mouse/i.v	Elevated levels of IgG2a and IgG1	[200]
	Priming with MOMP and secondary boost with DNA MOMP-ISCOM	i.m	Mouse/i.n	Elevated levels of IgG2a, IgA and IFNγ	[201]
	DNA MOMP plus GM-CSF, enterotoxins (*E. coli*) A and B	i.n + i.v	Pig/i.v	Vaccination induced significant protection against genital challenge Anti MOMP antibodies and low IL4 production	[202]
	OmpA	i.m	Pig/i.m		[203]
Bacterial ghosts	MOMP and PorB DNA plasmid	i.m	Mouse/i.v	High levels of IgG2a and IgA	[204]
	PmpD and PorB DNA plasmid	i.m	Mouse/i.v	High levels of IgG2a, IgA and IFNγ and low levels of IL5 (Th2)	[205]
Biodegradable polymers	rMOMP encapsulated in PLGA	s.c	Mouse	Elevated CD4+ and CD8+ T cells	[206, 207]
	Chitosan containing rMOMP DNA	i.m		Elevated IFNγ and IL12 and reduced IL4 and IL10	[208]
Vaccine from transgenic plants	MOMP introduced into *A. thaliana* and *D. carota*				[209]
Gas vesicles	Gene fragments coding for MOMP, OmcB and POMP loaded into *Halobacteria* –derived gas vesicles			Elicited Th1 cytokines in human foreskin fibroblasts	[210]

2.13 Adjuvants

Vaccines based exclusively on MOMP antigen alone, induce weak immune-protection against experimental genital chlamydial infection [211]. Therefore, the development of potent adjuvants is predicted to be the key to successful chlamydial vaccine development [152].

Adjuvants are substances combined with antigens to enhance the host immune response through the depot effect, antigen presentation or targeting, immune activation or modulation and cytotoxic lymphocyte infiltration. The protection may require a neutralising antibody response, interferon γ secretion by CD4+ T cells and cytotoxic CD8 lymphocyte response [212].

Toll like receptor agonists (TLR) are a group of immunopotentiators that promote maturation of the antigen presenting cells such as DCs (dendritic cells) [213]. TLR activation leads to activation and recruitment of the immune cells at the infection site, provision of adequate cytokines, chemokines and antimicrobial peptides, resulting in an overall adaptive immune response against conserved pathogen associated molecular patterns (PAMPs) [214] (Fig. 6).

Saponin-based adjuvants possess immunomodulatory abilities, including induction of balanced Th1/Th2 immune response, adequate antibody production [215] and cytotoxic CD8+ T lymphocytes [216] at the infection site through TLR-independent and IL-18 signalling pathways. ISCOMATRIX®, a saponin-based adjuvant, elicits rapid influx of innate cells to the draining lymph node [217]. This process enhances the cross and prolonged antigen presentation through antigen presenting cells (APCs) in the lymphatic channels [218] (Fig. 6).

Cationic adjuvant formulation (CAF01) consisting of DDA as a delivery vehicle and synthetic mycobacterial cordfactor as immunomodulatory. (Dimethyldioctadecylammonium (DDA) bromide and a, a'-trehalose 6, 6'-dibehenate (TDB), forms CAF01 [176] which has strong adjuvant activity through delaying release of antigen [217] with efficient memory T cell production [220]. It induces a Th1 biased immune response including Th17 response [221].

Aluminium containing adjuvants, widely used in human vaccines, can stimulate humoral immunity with a strong Th2 response [222]. It forms a depot effect at the injection site. The formation of particulate facilitated phagocytosis by antigen presenting cells [223]. The immunostimulatory property of aluminium salts depends on Nalp3 inflammasome activation [224], inducing pro-inflammatory cytokines, CD4+ T cell activation and antibody production [218] (Fig. 6).

2.14 Mechanism of action of adjuvant

2.14.1 TLR ligand agonist adjuvants

Toll like receptors (TLR) family recognise a wide range of microbial specific signature molecules, including nucleic acids, protein and lipid components of microbial membranes. Activation of TLRs results in a series of reactions followed by inflammation and innate immune response [225, 226]. Indeed, the diversity of the cascade reaction depends on the distinct TLR activated (summarised in table 5). This TLRs family are promising vaccine adjuvants, as they mainly induce Th1 biased immune response. Unmethylated CpG oligodeoxynucleotides (ODNs) is a TLR9 agonist adjuvant currently using in different animals models [226]. This adjuvant induces the production of pro-inflammatory cytokines

and overall eliciting a Th1-type immune response.TLR7 and TLR8 are single stranded RNA analogue, leading to type I IFN production and promoted Th1 response [225]. TLR3 is a double stranded RNA agonist as such poly inosinic:polycytidylic acid (poly I:C) that mimics viral RNA and exhibit as a potential vaccine adjuvant in different animal models [227-229]. In pigs, poly I: C was demonstrated to enhance the cell surface molecules which is thought to play the vital role for migrating the mature dendritic cells (DCs) to the lymphoid organs [230]. Nonetheless, poly I: C was shown to promote antigen specific CD4+ T cell response against malarial circumsporozoite protein [227].

2.14.2 Polyphosphazene based adjuvant

Polyphosphazenes (PCEP) are biodegradable polymers with an inorganic backbone consisting of alternative nitrogen and phosphorus atoms. Several laboratory animals' studies revealed that PCEP is potent immunological adjuvants that can enhance the quality, magnitude and duration of the immune responses against a wide range of vaccine antigens [231]. PCEP were shown to induce increased chemokines (CCL2, CXCL-10) and cytokines (IFN-γ, IL-4, IL-6, IL-8, IL-12) and antigen specific Ig (IgG1, IgG2) for a variety of bacterial and viral antigens [232-234]. Ideally PCEP has form micro-particles with antigen and enhance the stability, integrity of the vaccine during its formulation, processing and storage process [234]. In addition, this micro-particulate formulation effectively induces the antigen specific immune response in both plasma and mucosal compartment [235, 236]. PCEP was shown to significantly up-regulate antibody titers of IgG1 and IgG2a and induce a mixed Th1/Th2 immune response when co-administered with hepatitis B surface antigen (HBsAg) and influenza virus [232, 237]. Indeed, PCEP was shown to reduce the antigen requirement by 25-fold without affecting the quality and magnitude of the immune response [232].

2.14.3 Cationic host defense peptides (HDPs)

Cationic host defense peptides are small peptides that consist of positively charged and hydrophobic residues. The HDPs has been studied extensively in mouse models and majority of these studies focussed on immuno-modulation [238]. The immuno-modulatory properties of HDPs include (i) decrease the pro-inflammatory cytokines production induced against microbial signature molecules (ii) modulate the expression of chemokines (iii) induction of angiogenesis (iv) polymorphonuclear cells (PBMCs) activation (v) macrophage and leukocyte differentiation [239]. Initially the peptides bind either with surface receptors or the plasma membrane and then translocated inside the cellular compartment via various mechanisms [240]. After translocation, HDPs interact with intracellular receptors to generate multiple signal transduction pathways to elicit innate immune response [241].

2.14.4 Triple adjuvant combination

Combination adjuvants as dual and triple combinations are currently being tested in different animal's studies. However, the combination adjuvant can form complexes that become highly immunogenic and displayed a strong synergistic effect [242]. Novel combination adjuvants comprising HDP, PCEP and TLR ligand agonist has been assayed with a variety of vaccine antigens for a wide range of species including cattle, sheep, pigs, mice and cotton rats [241-245]. This vaccine approach shifted immune response to a Th1 type or mixed Th1/Th2 type [246]. In mice and pig's model, the *Bordetella pertussis* antigens in combination with CpG ODN, HDP and polyphosphazene was demonstrated to produce protective immune response. The longevity and magnitude of this immune response was effective following a single vaccination in the presence of maternal antibodies [247]. Recent vaccine trial utilising the chlamydial or influenza virus antigens, promoted strong innate and adaptive immune response in vaccinated animals [245, 248].

2.15 Safety of adjuvant

A wide variety of adjuvant have been utilised in different animals and human chlamydial vaccine trials (Table 5). Though a wide range of vaccine adjuvants elicit a strong immune response, most of them are too toxic for use in human vaccine formulations. For example, complete Freund's adjuvant (CFA) is a potent inducer of strong cellular immune response in mice, but it causes toxic effects in humans [225]. In koalas, TiterMax Gold has toxic side effects as it forms abscess at the injection side, though it has immune-modulatory properties (Table 6) [249].

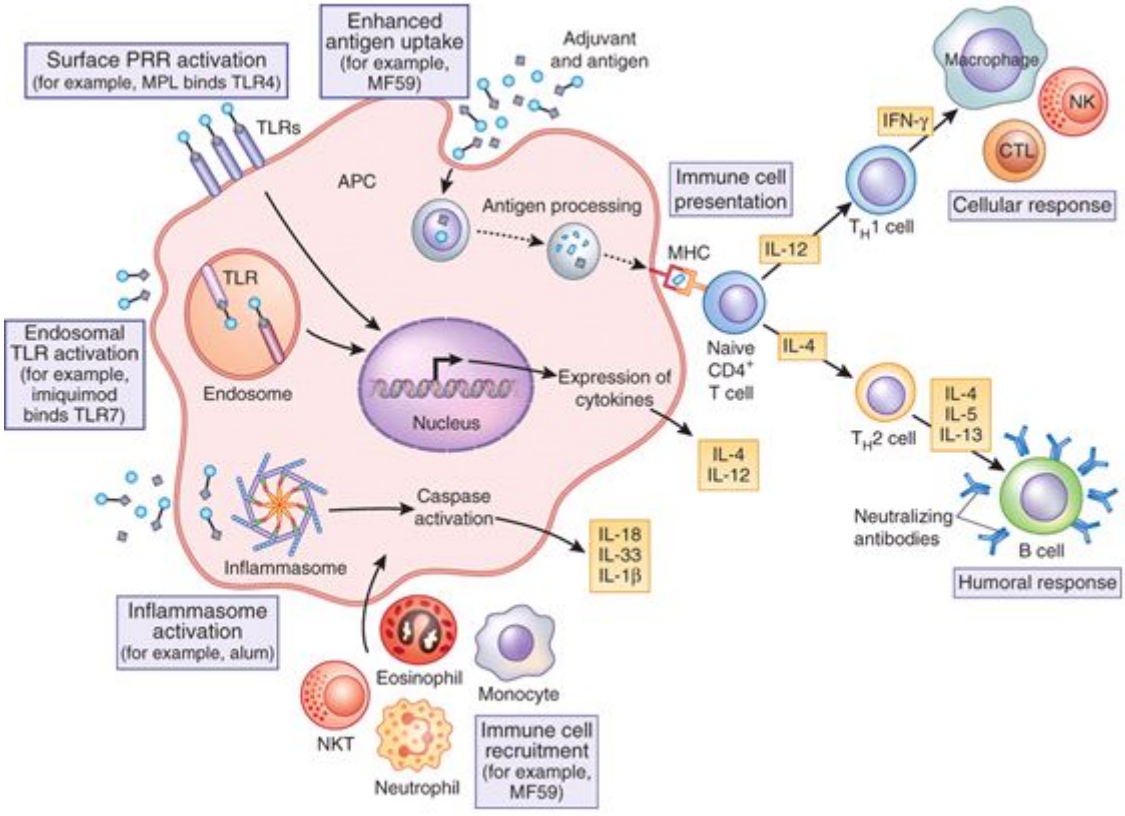
2.16 Stability and cost of adjuvants

In order to produce a large volume of vaccine for a larger group of animals, they must be available at low cost. In this regards, the potential vaccine adjuvants should be made with low cost production [250]. The ideal vaccine adjuvants can significantly reduce the amount of antigen required as well as it has antigen sparing properties. In addition, it is convenient to administer vaccine adjuvant that is stable at room temperature. Another important factor as simpler administers preferably the Holy Grail being single dose vaccine adjuvant [225].

Table 5. Summary of different adjuvant with their mode of action and specific immune response [251]

Adjuvant	**Class**	**Mechanism of action or receptor**	**Type of immune response**
dsRNA analogues (poly I:C)	Immunomodulatory molecule	TLR3	Antibody, Th1, CD8+ T cells
Lipid A analogues (MPL, RC529,GLA, E6020)	Immunomodulatory molecule	TLR4	Antibody, Th1
Flagellin	Immunomodulatory molecule	TLR5	Antibody, Th1, Th2
Imidazoquinolines (Imiquimod, R848)	Immunomodulatory molecule	TLR7 and TLR8	Antibody, Th1
CpG ODN	Immunomodulatory molecule	TLR9	Antibody, Th1, CD8+ T cells
Saponins (QS21)	Immunomodulatory molecule	Unknown	Antibody, Th1, Th2, CD8+ T cells
C-type lectin ligands (TDB)	Immunomodulatory molecule	Mincle, Nalp3	Antibody, Th1, Th17
CD1d ligands (α-galactosylceramide)	Immunomodulatory molecule	CD1d	Antibody, Th1, Th2, CD8+ NKT cells
Aluminium salts	Particulate formation	Nalp3, immunoreceptor tyrosine-based activation motif, antigen delivery	Antibody, Th2
Emulsions (MF59, AS03, AF03, SE)	Particulate formation	Immune cell recruitment, apoptosis-associated speck-like protein containing caspase recruitment domain, antigen uptake	Antibody, Th1, Th2
Virosomes	Particulate formation	Antigen delivery	Antibody, Th1, Th2
AS01 (MPL, QS21, liposomes)	Combination of immunomodulatory molecule and particulate formation	TLR4	Antibody, Th1, CD8+ T cells
AS02 (MPL, QS21, emulsion)	Combination of immunomodulatory molecule and particulate formation	TLR4	Antibody, Th1
AS04 (MPL, aluminium salts)	Combination of immunomodulatory molecule and particulate formation	TLR4	Antibody, Th1
AS15 (MPL, QS21, CpG, liposomes)	Combination of immunomodulatory molecule and particulate formation	TLR4 and TLR9	Antibody, Th1, CD8+ T cells
GLA-SE (GLA, emulsion)	Combination of immunomodulatory molecule and particulate formation	TLR4	Antibody, Th1
IC31 (CpG, cationic peptide)	Combination of immunomodulatory molecule and particulate formation	TLR9	Antibody, Th1, Th2, CD8+ T cells
CAF01 (TDB, cationic liposomes)	Combination of immunomodulatory molecule and particulate formation	Mincle, Antigen delivery	Antibody, Th1, CD8+ T cells
ISCOMs (saponin, phospholipids)	Combination of immunomodulatory molecule and particulate formation	Unknown	Antibody, Th1, Th2, CD8+ T cells

Fig 6. Mechanism of action of different classes of adjuvants. Some adjuvants can act through multiple pathways including antigen uptake, PPR signalling, inflammasome activation and recruitment of immune cells. (For example: Alum) [251]



2.17 Development of vaccine against chlamydiosis in koalas

Chlamydial MOMP is the best studied immune-protective antigen to date [67, 252]. Nevertheless, vaccination with MOMP does have its limitations. The surface exposed variable domains of MOMP are genetically diverse. Initially in the process of vaccine design, our group utilized MOMP as the vaccine antigen along with NrdB and CT512 with three different adjuvants namely Alhydrogel, TiterMax Gold and Immune Stimulating Complex (ISC) in koalas [249]. Recombinant NrdB was used to prime CD4+ T cells to produce both anti-*Chlamydia* antibodies and a lymphocyte response. This antigen has the advantage that it is highly conserved across many chlamydial species. CT512 is an immunogenic antigen, originally developed from outer membrane protein (*omp85*) of *C. muridarum*, and elicits partial protection against genital chlamydial infection in mouse model [253]. This preliminary study showed that a multi-subunit vaccine is feasible in koalas against chlamydial infections. The adjuvant, ISC is composed of purified saponin complex with cholesterol and phospho-lipid and has shown cytotoxic T-cell response in different animal models [201]. Vaccination of koalas with rMOMP plus ISC adjuvant induced a strong cellular and humoral response in several trials [249, 254-256]. These studies showed a multi-subunit rMOMP based vaccine could provide immune-protection against chlamydial infection in koalas.

The major issue of chlamydial infection in humans is the asymptomatic nature of the infection. This is also a problem in koalas with molecular studies showing high prevalence rates in the absence of disease (reviewed in Polkinghorne et al. [75]). Interestingly, Wan et al. [257] showed that high infection loads can be found in asymptomatic animals [257]. These animals might be a source of infection for future transmission of *Chlamydia* in wild koalas.

Therefore, the importance of producing a vaccine that elicits immune response in healthy as well as in diseased koalas is significantly important.

Due to the diversified nature of the MOMP protein [258], it was unexpected to have a similar form of immune response in koalas that have either received a single or multiple dose of MOMP vaccine. Surprisingly, single MOMP vaccinated koalas were able to recognize the homologous as well the heterologous strains of koala *C. pecorum* [255]. This study is very promising as it illustrates the feasibility of developing an effective recombinant MOMP-based *C. pecorum* vaccine against a wide range of circulating strains. Nonetheless, a recent study utilising the Pepscan method (www.pepscan.com) was able to identify the B cell epitopes across the MOMP protein of four *C. pecorum* strains. This study revealed that vaccine induced epitopes were distinct from natural infection [256]. Most of the epitopes were in the conserved domain, suggesting the cross reactive nature of the previously described immune response with single rMOMP protein [258].

Table 6. The vaccination profile for koala's (Literature thus far)

Antigen	Adjuvant	Immune response	Reference
MOMP, NrdB and TC0512	Alhydrogel	IgG had significant response in genital tract up to 158 days	[249]
		Significant Peripheral blood mononuclear cell (PBMC) proliferation	
MOMP, NrdB and TC0512	Immuno-stimulating complex (ISC)	Sustained plasma IgG up to 1 year	[249]
MOMP, NrdB and TC0512	TiterMax Gold	Abscess in the inoculation site	[249]
		Minimal IgG response up to 102 days	
MOMP and NrdB	Immuno-stimulating complex (ISC)	Sustained IgG in the plasma and eyes secretion up to 140 days	[254]
MOMP monovalent	Immuno-stimulating complex (ISC)	Sustained plasma IgG for MOMP A up to 21 wks	[255]
		Sustained plasma IgG for MOMP F up to 21 wks	
		Plasma IgG response up to 10 wks for MOMP G	
MOMP polyvalent	Immuno-stimulating complex (ISC)	Significant IgG up to 10 wks and moderately up to 21 wks	[255]

While a chlamydial vaccine development in koalas looks promising, we still lack species specific immunological reagents due to our lack of understanding of the koala immune response against infection or vaccination. Recently Mathew et al. [259] provided some knowledge in koala immunology by optimising and developing the koala specific quantitative real time PCR assays for measuring the important Th1 cytokine, IFN-γ .In addition, they identified IL-17A gene expression in diseased animals as a key immune marker for chlamydial disease severity and pathogenesis [260]. Furthermore, they characterised the expression of anti-inflammatory (TNF-α and IL-10) cytokine in a cohort of diseased koalas [261].

2.18 Concluding remarks

A *Chlamydia* vaccine is the most appropriate and realistic approach against the widespread infections that continue to be present in koalas. Early human trachoma vaccine trials utilising whole inactivated *Chlamydia*, resulted in adverse disease pathology in recipients [127]. Since then, immune-protective subunit antigens have been the focus of anti-chlamydial vaccine designs. While MOMP of *Chlamydia* is the best studied antigen to date, identification of novel and immune effective antigens combined with appropriate adjuvants that could replace the multi-dose strategy through a simpler single vaccine dose would certainly be beneficial to the welfare of koalas.

2.19 References

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Chapter 3: 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

3.1 Statement of contribution of co-authors for thesis by published paper

The authors listed below have certified that:

1. They meet the criteria for authorship in that they have participated in the conception, or execution, or interpretation, of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to (a) granting bodies, and (b) the editor or publisher of Vaccine and
5. They agree to the use of the publication in the student's thesis and its publication on the QUT ePrints database consistent with any limitations set by publisher requirements.

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Contributor	Statement
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Courtney Waugh	Contribution to critical reading and editing of the manuscript and contributed to the collaboration
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3.2 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 utilised 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 immunising 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.

Key words: *Chlamydia*, Koala, Single dose, Vaccine, Adjuvant

3.3 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 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 wks) 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 immunisation for induction of anti-chlamydial immunity in the koala.

3.4 Materials and methods

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′-Kpnl 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. Lysed cells were incubated with TALON metal affinity resin at 4° C for 1 hr 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.

Adjuvant

The combination adjuvant consists of polyposphazine (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.

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).

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). Blood samples were collected at 0 weeks (pre-immunized), 6, 10, 14, 21, 41 and 54 weeks. UGT and ocular swabs were collected at 0 weeks (pre-immunized), 6, 10, 21, 41 and 54 weeks time points.

Measurement of anti-MOMP lymphocyte proliferation response

Lymphocyte proliferation responses were assayed as per Carey et al.[16] except that UV-irradiated *C. pecorum* G elementary bodies (EBs) were used for *in vitro* stimulation of PBMCs. PBMCs were collected from blood samples at 0 (pre-immunized), 6, 21 and 54

weeks after the first immunization. PBMCs were isolated on Ficoll gradients, labelled with Carboxyfluorescein succinimidyl ester (CFSE) then stimulated with rMOMP, UV-inactivated *C. pecorum* EBs or Concanavalin A (ConA) as a positive stimulator, and no antigen as a negative control. Proliferation of PBMCs was determined using a Beckman Coulter flow cytometer (FC500, Gladesville, NSW, Australia). Proliferation was expressed as the % of PBMCs that had undergone > 3 cell divisions. The mean fluorescent intensity of individual CFSE peaks, representing cycles of cell division, was analysed using ModFit software as described in Lyons and Doherty [25].

Enzyme linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISAs) were performed as per Kollipara et al.[15] at 0 (pre-immunized), 6,10, 14, 21, 41 and 54 weeks for plasma and 0 (pre-immunized), 6, 10, 21, 41 and 54 weeks for swab samples. To obtain material from the swabs, the swabs were were collected into the protease inhibitor, PMSF plus buffer, vortexed vigorously, centrifuged and the supernatant analysed for antibodies by ELISA.

In vitro Chlamydia neutralization assay

In vitro neutralisation assays were performed using koala plasma and mucosal secretions (UGT and ocular) collected at 0, 6, 10, 21 and 54 weeks post immunization according to Kollipara et al. [15]. All samples were diluted 1:10 prior to testing. For each assay, *C. pecorum* genotype G purified EBs (50,000 IFU) were mixed with the diluted plasma or secretions and incubated at 37C for 30 min prior to inoculation onto cell monolayers for assay of residual infectivity. Pre-immunisation plasma, ocular and UGT secretions were

collected from each individual animal pre-vaccination and were used to establish the background levels for each koala. The individual background level was then subtracted from post-vaccination neutralization levels for each individual to get the actual neutralization effect.

Statistics

All statistical analyses were performed using Graph-Pad Prism version 6 (Graph pad Software, LaJolla, CA, USA). Data are presented as mean ± SD from triplicate assays. Data between cohorts was analysed using one –way ANOVA Kruskal-Wallis (non-parametric) tests. The P value for significance was set at ≤ 0.05.

3.5 Results

Vaccine safety

There were no adverse effects following administration of the vaccine in any of the six koalas (such as swelling or abscess formation around the injection site) immediately after immunization, or for the entire study period. There was no evidence of clinical illness from the vaccine for the 54 weeks of the trial (data not shown).

Cellular immunity

CFSE dye-dilution assays demonstrated significant PBMC proliferation following *in vitro* stimulation with rMOMP (Fig.1) and UV-inactivated EBs (Fig. 2) at the 6, 21 and 54 weeks post-immunization time-points, compared to pre-immunization levels. Recombinant MOMP induced the highest proliferation rates (20.3% in single dose; 20.8% in double dose) (p value 0.842)(Fig. 1) compared to UV inactivated EBs (9.5% in single dose; 13.4% in double dose) (Fig. 2) at the six week time point. At 21 weeks post-immunization, PBMCs had slightly reduced proliferation in both vaccine cohorts compared to the six-week time point for both rMOMP (10.4% in single dose; 14.1% in double dose) and EBs (9.3% in single dose; 11.4% in double dose). At 54 weeks post immunization, PBMCs were still proliferating in response to both rMOMP (9.7% in single dose; 9.8% in double dose) (Fig. 1) and EBs (6.04% in single dose; 5.9% in double dose) (Fig. 2). No statistically significant differences were observed in PBMC proliferation levels between the single and double vaccine groups at any time point.

Humoral immunity at systemic and mucosal sites

MOMP-specific IgG antibody in plasma (Fig. 3) and mucosal secretions (Fig. 4) were determined by ELISA. All pre-immunizations samples were negative for MOMP IgG. Plasma IgG levels peaked at 41 weeks for both groups of koalas (EPT: End Point Titer; 2-3 $x10^6$), before beginning to plateau or decrease slightly by week 54. No significant differences in plasma EPT were observed between koalas in the single and double dose vaccine groups at the end period (54 weeks). In UGT secretions (Fig. 4 A), the IgG EPT in both groups peaked at 10 weeks post-immunization (EPT 1.5-3.2$x10^2$). Ocular antibody EPT (Fig. 4 B and C) were similar for both eyes and reached a peak by week 21 (EPT 1.0-2.2$x10^2$) and then declined by week 41. There were no significant differences in IgG levels in UGT and ocular secretions between the single and double dose vaccine groups.

Neutralizing antibodies in plasma, vaginal and ocular secretions

Fig. 5 A, B and C demonstrate the percent neutralization in plasma, UGT and ocular secretions compared to pre-immunization samples. All samples were tested at a 1:10 dilution. The increase in plasma neutralising antibody (Fig. 5A) peaked at 21 weeks (69% in single dose; 70% in double dose) and had declined by week 54 (57% in single dose; 62% in double dose). Neutralizing antibodies in the UGT secretions increased (17% in single dose; 25% in double dose) (Fig. 5 B) in both groups, peaking at 21 weeks then declining out to 54 weeks. Neutralizing antibodies in ocular secretions (Fig. 5C) ranged from 4-25%, peaking at 21 weeks post immunization in both cohorts.

3.6 Discussion

This study evaluated a novel vaccine consisting of a chlamydial rMOMP combined with a poly I:C, polyphosphazine (PCEP) and a synthetic host defense peptide adjuvant, given subcutaneously as either a single or double dose, to healthy female koalas. We have recently developed a rMOMP-based vaccine that induces strong cell-mediated and humoral immune responses following two or three vaccinations. This vaccine was effective in naturally infected and diseased koalas, as well as in healthy individuals [15] and elicited cross neutralizing antibodies that were able to neutralize multiple MOMP genotypes of *C. pecorum in vitro* [14]. While these results are very promising, the deployment of this vaccine to wild koalas in care or in the field will be problematic as the use of injectable vaccines in wild koala populations requires capture and restraint of wild animals, or a prolonged stay in a koala hospital or wildlife carer facility. Beyond the logistical and economic considerations associated with the vaccine delivery schedule, capture of a wild koala is a stressful and traumatic process for each animal and hence alternative single dose regimes would be ideally suited for vaccination of wild animals.

The adjuvant components used in this single-dose vaccine experiment were chosen based on the immunomodulatory effects of each of the individual components, which in combination, should induce the type of immune response required to provide protection against *Chlamydia*, namely a strong cell-mediated response as well as a humoral response [26]. Poly I:C is a synthetic dsRNA and a TLR3 ligand. TLR3 is found in the endosomal compartment of a wide variety of cell types including monocytes, macrophages, Langerhans cells, myeloid dendritic cells, as well some fibroblasts and epithelial cells. Binding of poly I:C by TLR3 results in the production of type 1 interferons (IFNα and IFNβ) as well as pro-inflammatory cytokines

such as IL-12 and IL-6. TLR3 ligation by poly I:C also induces maturation of dendritic cells and the transcription of many interferon-regulated genes (IRG). Poly I:C can also trigger innate anti-viral immunity by binding to the retinoic acid inducible gene 1 (RIG-1) receptor, which also elicits production of type 1 interferons and pro-inflammatory cytokines. Poly I: C has been used as an adjuvant in a wide range of animal species (monkey, mice, pigs) to target diseases as such papilloma virus, foot and mouth disease, influenza and tuberculosis [27-29].

Polyphosphazenes are high molecular weight, water soluble polymers consisting of a backbone of alternating phosphorous and nitrogen atoms with organic side chains attached to each phosphorous that have been shown to possess strong adjuvant activity. The best-studied polyphosphazene adjuvant is PCEP. Following intramuscular injection PCEP induces the expression of many innate immune genes ("adjuvant core response genes")[31] including chemokines, inflammatory cytokines, pattern recognition receptors (PRR), interferon-regulated genes, adhesion molecules and antigen presentation-associated genes. Importantly, PCEP activated the NLRP3 inflammasome, resulting in production of caspase-1 dependent cytokines IL-1β and IL-18. Thus PCEP creates a strong immune-stimulatory environment at the injection site, which likely contributes to its adjuvant activity together with the formation of water-soluble protein polymer complexes with antigen, which facilitates antigen processing and may provide a depot effect. In mouse studies, PCEP induces recruitment of neutrophils, macrophages and dendritic cells to the injection site [20], is a potent inducer of mixed Th1/Th2 responses when administered with influenza antigen [30] and elicits a strong mucosal IgA response when delivered by nasal, vaginal and pulmonary routes [22].

Cationic host defense peptides (HDP) are small peptides that contain mainly positively charged and hydrophobic residues. They are abundant in eukaryotes as well as being found in bacteria and appear to be highly conserved across species. They were originally isolated because of their antimicrobial activity, having varying direct toxic effects against bacteria, viruses, fungi and parasites. With regard to potential adjuvant activity, HDP have multiple immunostimulatory properties including leukocyte recruitment and activation, enhanced pro-inflammatory cytokine production, increased co-stimulatory molecule expression on APCs and increased phagocytic activity. When administered together with CpG the HH2 HDP elicited strong mixed Th1/Th17 responses that protected against chlamydial infection in a mouse model [31].

The combination of poly I:C, PCEP and HDP (HH2) has proven to be an extremely potent adjuvant in mouse and porcine infection models, where a single immunization resulted in high titers of circulating antibodies ($>10^6$) that were extremely long lasting. This combination adjuvant was effective even in neonates and was unaffected by circulating maternal antibody.

Using this novel adjuvant mix, the results of the current study suggest that vaccinated koalas developed strong lymphoproliferative responses to our vaccine. MOMP-specific PBMC proliferation was detectable for over a year in animals immunized with both single and double doses of the vaccine. Importantly, vaccinated koalas responded to both the vaccinating antigen (rMOMP), as well as the native whole chlamydial EB. A strong Th1 response has been shown to be essential for protection against *Chlamydia* in a murine model [32]. While we do not yet have the reagents to identify CD4 cells in the koala, the induction of long-lived cell-mediated responses are promising. Furthermore, the ability of this combination adjuvant

to induce strong Th1/Th17 responses and mixed Th1/Th2 responses in mice and pigs [30,33] makes it highly likely that vaccinated koalas will mount a strong and successful anti-*Chlamydia* Th1 response, although this needs to be confirmed.

When comparing the results of this work to our previous multi-dose trials, the present single dose combination adjuvant appears to produce similar immune responses to those previously described in koalas receiving multiple doses of our rMOMP-based vaccine [14, 15]. The adjuvant component, PCEP, may be partially responsible for the antibody in mucosal secretions since it has been shown to enhance mucosal antibody (IgA and IgG) responses [34] in mouse models. Though we have no koala IgA reagent to measure this antibody at mucosal surfaces, currently, the presence of IgG responses in mucosal secretions is a positive sign. The degree of neutralization achieved in this study (60-70% for plasma) was similar to that obtained in a previous study using rMOMP and ISC adjuvant (50-90%) [15, 16].

In summary, the current study has shown that the novel single dose vaccine using a poly I:C, PCEP, HDP combination adjuvant is capable of inducing significant cellular and humoral immune responses in koalas for an extended time period (54 weeks). Although the animal numbers were small, the results are promising and suggest that a single shot vaccine may represent an effective approach for protecting wild koalas against chlamydial infection while minimising potential capture-related stress associated with booster immunizations. Future work will evaluate this single dose antigen: adjuvant vaccine in animals under field conditions.

3.7 Acknowledgements

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Conflict of interest: The authors declare that they have no conflict of interest.

3.8 References

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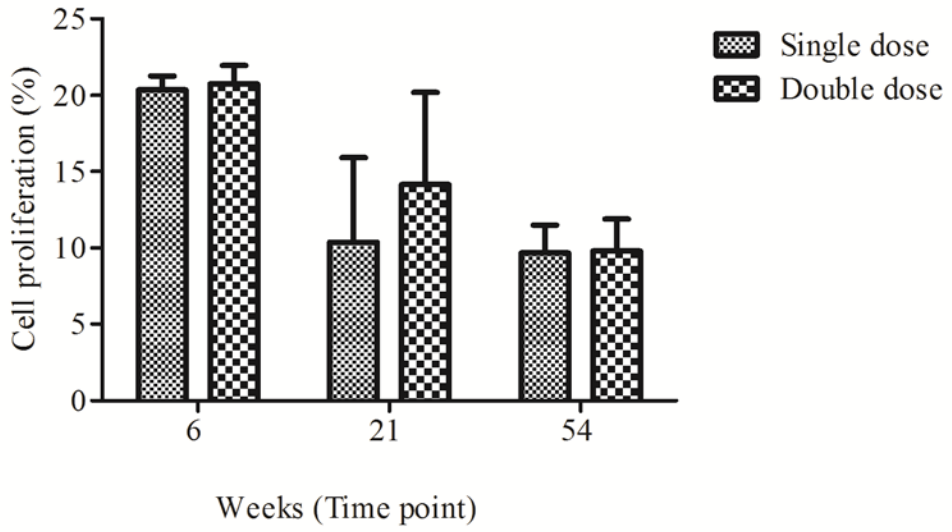

Fig. 1. Lymphocyte proliferation assay (LPA): PBMCs were isolated from koalas at 6, 21 and 54 weeks post-immunization, labelled with CFSE and stimulated with rMOMP. Results are presented as the percentage of cells that have undergone three or more cell divisions and are expressed as the mean ± SD (n = 3 animals)

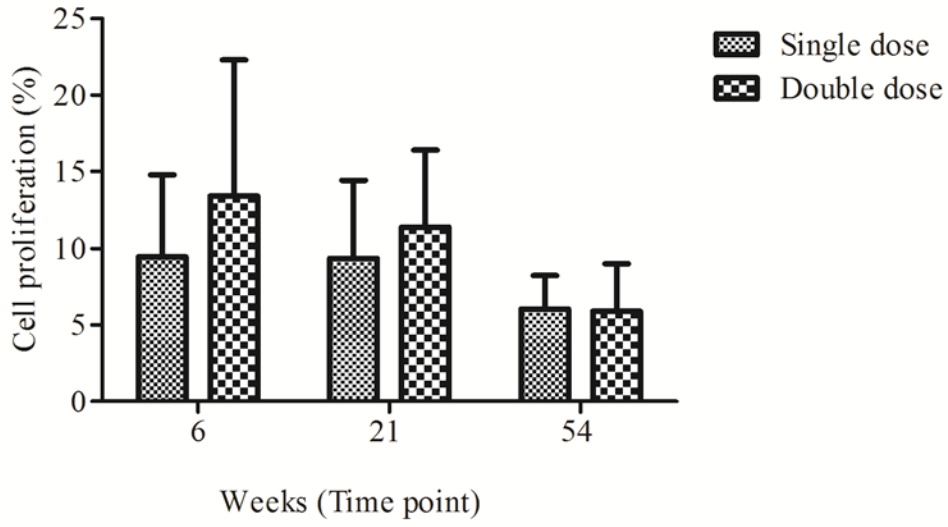

Fig. 2. Lymphocyte proliferation assay (LPA): PBMCs were isolated from koalas at 6, 21 and 54 weeks post-immunization, labelled with CFSE and stimulated with UV-inactivated *C. pecorum* EBs. Results are presented as the percentage of cells that have undergone three or more cell divisions and are expressed as the mean $\pm$ SD (n = 3 animals)

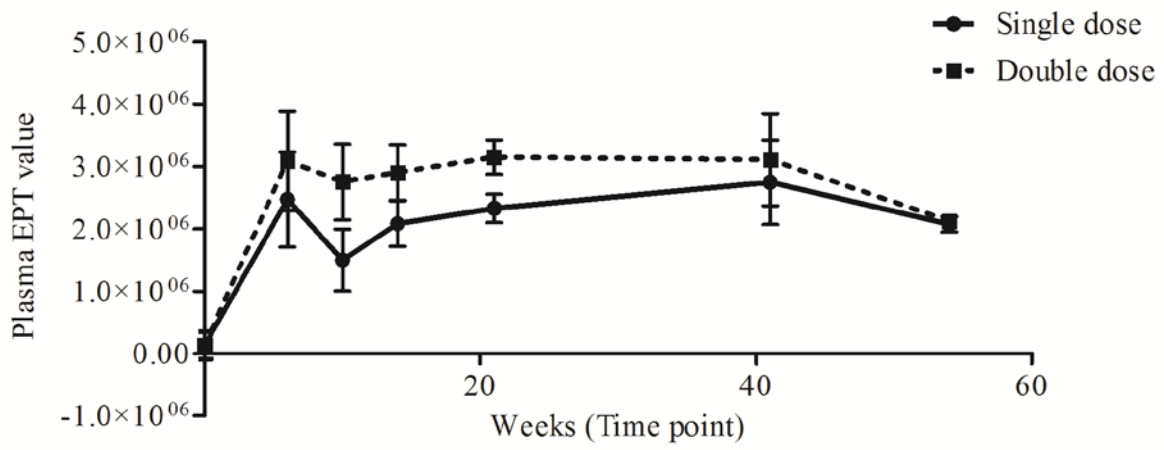

Fig. 3. rMOMP-specific IgG in koala plasma: rMOMP-specific IgG in plasma of immunized koalas was assayed by ELISA at 0, 6, 10, 14, 21, 41 and 54 weeks post immunization. IgG levels are expressed as end-point titers and represent the mean $\pm$ SD of three animals per group. EPT at 54 weeks did not differ significantly between koalas immunized once or twice.

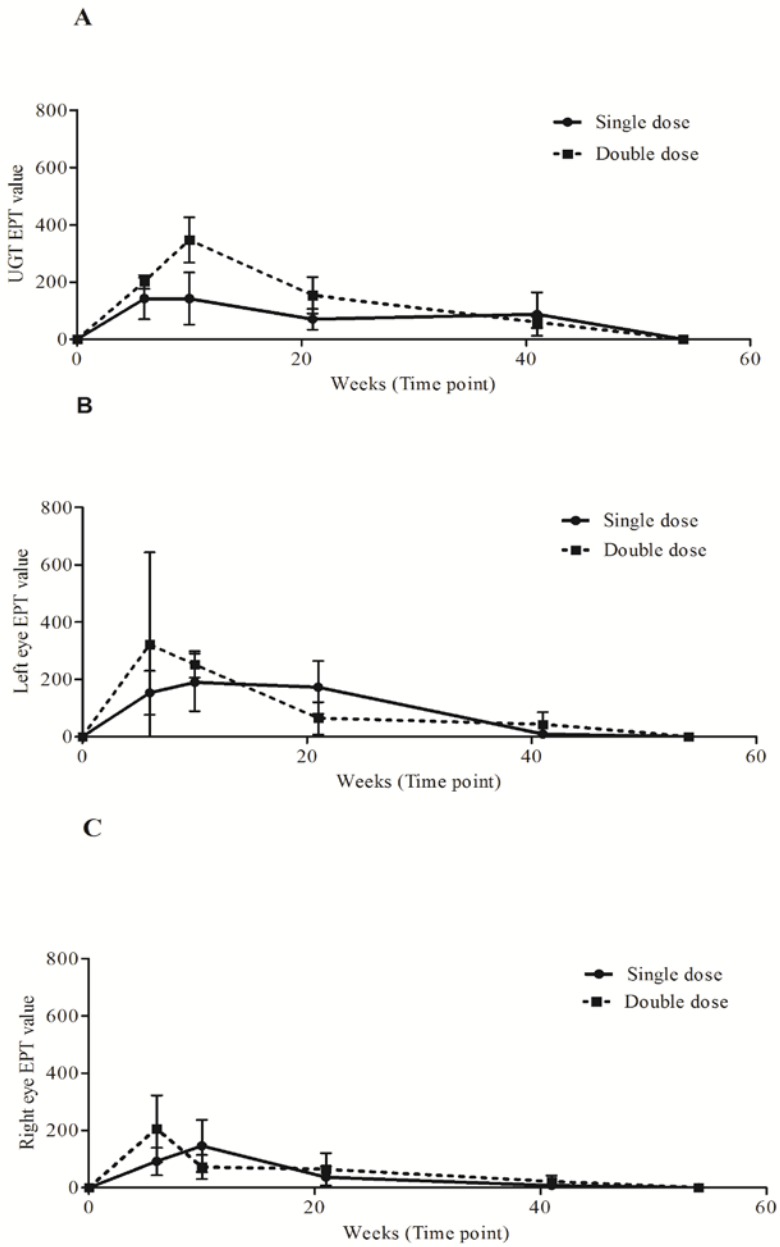

Fig. 4. rMOMP-specific IgG in koala mucosal secretions: rMOMP-specific IgG in urogenital secretions (A), left eye swabs (B) and right eye swabs (C) of immunized koalas were assayed by ELISA at 0, 6, 10, 21, 41 and 54 weeks post immunization. IgG levels are expressed as end-point titers and represent the mean + SD of three animals per group. There were no statistically significant differences in EPT in samples collected from koalas that had been immunized once or twice.

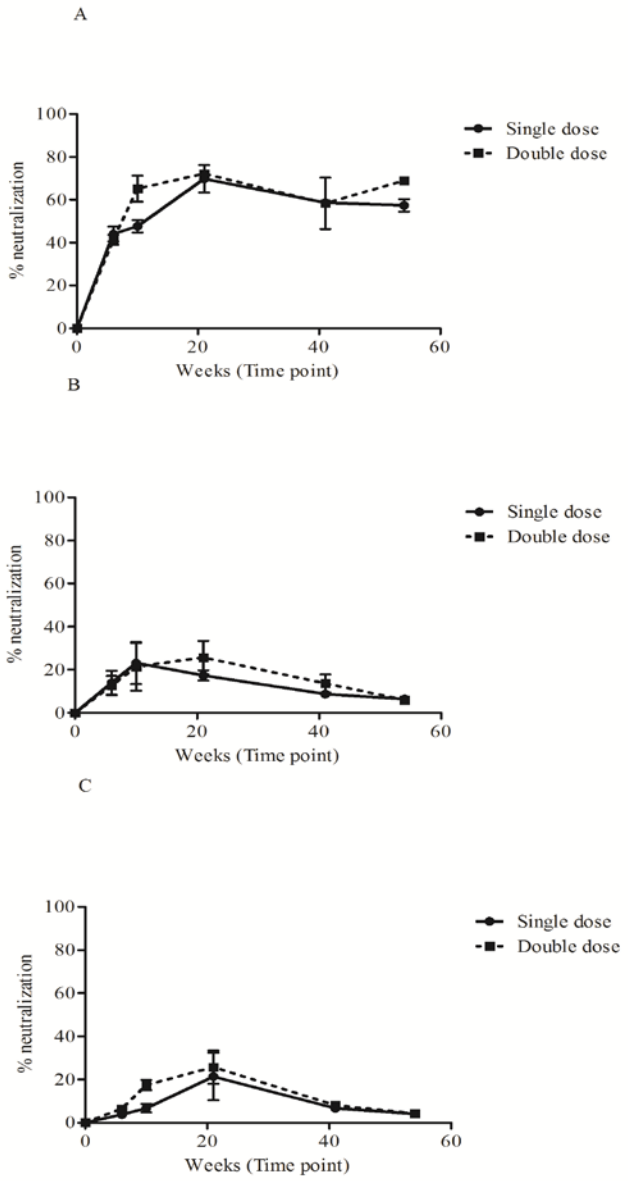

Fig. 5. Percentage of vaccine induced *C. pecorum* neutralizing antibodies in plasma (A), UGT secretions (B) and ocular secretions (C). All samples were diluted 1:10 and *C. pecorum* EBs (50,000 IFU) were added to samples and results are expressed as the percentage in neutralization of immune samples compared to that of the pre-immune samples. Results are expressed as the mean $\pm$ SD of three animals per group. There were no significant differences between samples collected from animals immunized once or twice.

Chapter 4: Humoral immune responses in koalas (*Phascolarctos cinereus*) either naturally infected with *Chlamydia pecorum* or following administration of a recombinant chlamydial major outer membrane protein vaccine

4.1 Statement of contribution of co-authors for thesis by published paper

The authors listed below have certified that:

1. They meet the criteria for authorship in that they have participated in the conception, or execution, or interpretation, of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to (a) granting bodies, and (b) the editor or publisher of Vaccine and
5. They agree to the use of the publication in the student's thesis and its publication on the QUT ePrints database consistent with any limitations set by publisher requirements.

In the case of this chapter:

Khan, S.A., Polkinghorne, A., Waugh, C., Hanger, J., Loader, J., Beagley, K., and Timms, P. (2016). Humoral immune responses in koalas (*Phascolarctos cinereus*) either naturally infected with *Chlamydia pecorum* or following administration of a recombinant chlamydial major outer membrane protein vaccine. Vaccine. 34, 775-782.

Contributor	Statement
Shahneaz Ali Khan	Contributed to the project design and over all implementation, conducted the majority of the laboratory work in the project, conducted the analysis and interpretation of the data and wrote the manuscript
Signature	
Date	
Adam Polkinghorne	Contribution to critical reading and editing of the manuscript and contributed to the overall collaboration
Courtney Waugh	Contributed the laboratory work for qPCR data and involved in reading and editing the manuscript
Jon Hanger	Veterinary expertise and contributed to thorough clinical examination and sampling of koalas in this field study
Jo Loader	Veterinary expertise and contributed to the sampling of koalas and involved in the collaboration and approval of this study
Kenneth Beagley	Contributed to the project design, critical reading and editing of the manuscript
Peter Timms	Designed the initial research plan; involved in experimental planning , design and securing funding; major contributor in collaborating with the industry partners; critical reading, editing and approved the manuscript

Principal Supervisor Confirmation

I have sighted email or other correspondence from all co-authors confirming their certifying authorship.

PETER TIMMS		3/5/2016
Name	Signature	Date

4.2 Abstract

The development of a vaccine is a key strategy to combat the widespread and debilitating effects of chlamydial infection in koalas. One such vaccine in development uses recombinant chlamydial major outer membrane protein (rMOMP) as an antigen and has shown promising results in several koala trials. Previous chlamydial vaccine studies, primarily in the mouse model, suggest that both cell mediated and antibody responses will be required for adequate protection. Recently, the important protective role of antibodies has been highlighted. In our current study, we conducted a detailed analysis of the antibody mediated immune response in koalas that are either (a) naturally infected, and/or (b) had received an rMOMP vaccine. Firstly, we observed that naturally infected koalas had very low levels of *C. pecorum* specific neutralising antibodies. A strong correlation between low IgG total titers/neutralising antibody levels, and higher *C. pecorum* infection load was also observed in these naturally infected animals. In vaccinated koalas, we showed that the vaccine was able to boost the humoral immune response by inducing strong levels of *C. pecorum* specific neutralising antibodies. A detailed characterisation of the MOMP epitope response was also performed in naturally infected and vaccinated koalas using a PepScan epitope approach. This analysis identified unique sets of MOMP epitope antibodies between naturally infected non-protected and diseased koalas, versus vaccinated koalas, with the latter group of animals producing a unique set of specific epitope-directed antibodies that we demonstrated were responsible for the *in vitro* neutralisation activity. Together, these results show the importance of antibodies in chlamydial infection and immunity following vaccination in the koala.

Key words: Koala, *Chlamydia*, Humoral immunity, Vaccine

4.3 Introduction

While the koala is facing a number of threats to its long-term survival (habitat destruction, road accidents, dog attacks, bush fire)[1-3], disease caused by the obligate intracellular bacterial pathogen, C*hlamydia* is considered to be one of the more serious issues and one that is amenable to intervention [4]. For this reason, the development of a chlamydial vaccine is a major priority. Chlamydial vaccine research in animal models indicates that a combination of cell-mediated and antibody responses are required for effective protection [5]. Antibodies, particularly against surface antigens, are thought to have a key neutralising role, but they are also considered to play a role in inflammatory disease processes [6]. Antigen-antibody complexes have a direct effect in the inflammatory pathway and initiate the cellular immune response via complement activation and cross-linking of Fc receptors (FcR) to promote phagocytosis [7]. The presence of mucosal antibodies has been found to be linked with decreased chlamydial shedding in women with genital tract *C.trachomatis* infections [8]. Moreover, B cell-deficient mice were prone to infection with secondary chlamydial infections. Interestingly, B cells promote antigen-specific T cell responses and bacterial dissemination in genital *Chlamydia muridarum* infection [9]. In addition, B cells play a key role in the initiation of T cell responses for *C. trachomatis* infections in the murine model [10].

Over the last five years our group has been developing a *Chlamydia pecorum* vaccine for koalas [11-14]. We have mainly focused on the chlamydial major outer membrane protein (MOMP) as the target antigen, including three MOMP variants in our most recent trials. Immunisation with recombinant MOMP (rMOMP) protein can lead to a strong antibody, as well as a specific lymphocyte response, up to at least one year post-vaccination [11-13].

While this work has shown encouraging signs in terms of our ability to induce specific and functional antibody responses in vaccinated animals, relatively little is still known about the role of antibodies in naturally-infected animals. Specifically, it is currently unknown, (a) whether naturally-infected koalas produce antibodies of sufficient quantity and specificity to control current infections and protect against future infections, and (b) whether vaccination can induce antibodies of comparable or higher titers and specificity than a natural infection. In the current study, we aimed to address these questions by studying the immune responses of a wild population of koalas, including both infected and uninfected individuals. Subgroups of either also received the prototype rMOMP vaccine. We characterised (a) total anti-*Chlamydia* IgG response against rMOMP protein as well as whole chlamydial elementary bodies (EBs), (b) *in vitro* neutralisation activity, and (c) the epitope specificity of the antibody response using a Pepscan approach.

4.4 Materials and Methods

Animals

The animals analysed for this study were part of a population of koalas inhabiting the Moreton Bay Region (MBR) of South-East Queensland, Australia. A sub-sample of this population (n = 30) had also been vaccinated with our rMOMP vaccine (described below) as part of a larger ongoing vaccine study. For the purposes of the current study, a total of 20 wild koalas were monitored from this MBR population over a 6-month period. The koalas were divided into four cohorts (each consisting of five koalas) on the basis of *C. pecorum* infection load by qPCR at the initial stage of enrolment. The groups were, (a) infected with *Chlamydia*/no vaccination (Bubbles, Cougar, Karen, Coco, and Mango), (b) infected with *Chlamydia*/vaccinated (Tash, Bev, Old Bean, Fiona, Poppy), (c) No *Chlamydia* infection/no

vaccination (Kev, Teena, Phill, Red Queen, Gauthier), (d) No *Chlamydia* infection/vaccinated (Robyn, Pepper, Maya, Hunky Harry, Randall). All work was conducted under permission from Queensland University of Technology's Animal Ethics Committee (Permit # 1200000122).

Vaccine

Koalas recruited into the vaccine cohorts received three doses of a vaccine via the subcutaneous route [15]. Koala *C. pecorum*- specific MOMP proteins were expressed and purified as per Kollipara et al.[11]. The purified products were used for vaccination and ELISA assays.

Sample collection

The koalas were captured on 0, 2 and 6-month time points for sampling (blood from the cephalic vein and ocular and urogenital tract swabs) and a full health assessment. Swab samples were also collected from the conjunctival and urogenital (UGT) mucosa [14] and stored immediately at -20° C until analysis.

Screening of *C. pecorum* infections

Ocular and urogenital tract (UGT) swab samples from all koalas were screened for the presence of *C. pecorum* infection by a species-specific 16S rRNA quantitative PCR (qPCR) as per Marsh et al. [16].

Koala *C. pecorum*-specific IgG ELISA

Enzyme-linked immunosorbent assays were performed using either (a) rMOMP protein or (b) UV-inactivated whole EBs as per Kollipara et al. [11] on the plasma samples at the 0, 2 and 6-month time points.

C. pecorum in vitro neutralisation assay

In vitro neutralisation assays were performed on all plasma samples as per Kollipara et al. [12]. All plasma samples were diluted at 1:10 prior to assay. The background neutralisation was determined by using koala plasma that had no infection. Actual neutralisation was then determined by subtracting this background from each individual to determine the final neutralisation. The results were expressed as fold-change neutralisation.

MOMP epitope mapping by Pepscan ELISA

The Biotinylated peptide ELISA was performed using plasma samples as per Kollipara et al.[17] to identify the specific MOMP peptide recognised by (a) vaccinated koalas and (b) naturally-infected koalas. Briefly, we designed 88, 15-mer peptides (overlapping by 9 amino acids) that spanned the full length of koala *C. pecorum* MOMP. The wells of 96 (previously coated with streptavidin) were coated with each individual peptide at a concentration of 2μg/well in PBST (Phosphate buffer solution-Tween20) and incubated for 2 hrs at room temperature. Post incubation, the wells were washed 3x with PBST and coated with individual plasma samples at 1:1000 dilution for overnight incubation at 4° C followed by 4xPBST wash. The plates were then incubated with the secondary and tertiary antibody with sheep anti-koala IgG (1:4000 dilution) and HRP-labelled rabbit anti-sheep IgG (1:1000 dilution) (Southern Biotech/In vitro Technologies, Cleveland, Australia) respectively. After 1 hr incubation, the plates were washed with PBS (Phosphate buffer solution) and ABTS [2,

2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), Southern Biotech, Alabama, USA] solution was added and incubated for 10 mins to observe the greenish color development. The optical density was measured at 405nm wavelength (Bio-Rad, North Ryde, Australia). In subsequent experiments, we utilised just the positive peptides to coat the streptavidin plate at a concentration of 2μg/well and performed the standard ELISA as described.

Characterisation of the specific anti-epitope antibodies responsible for *Chlamydia in vitro* neutralisation

Once we had identified the epitope specificity of plasma from the post-vaccinated koalas, we wanted to determine if the *in vitro* neutralisation capacity of the plasma was explained by the antibodies to these epitopes. We coated the plates just with the specific peptides (4, 28, 41, and 42) at a concentration of 2μg/well and incubated at room temperature for 2 hrs. Post-incubation, the plates were washed with 3x PBST and incubated with the positive plasma samples at 4° C overnight. This step resulted in removal of the antibodies to the selected key peptides. Post-incubation plasma was collected and used in the biotinylated peptide ELISA as described above. We then measured the *C. pecorum*-specific neutralising antibodies for the pre- and post-adsorption plasma, as described above.

Statistics

Statistical analyses were performed using Graph-Pad Prism version 6 (Graph Pad Software, La Jolla, CA, USA) and SPSS.22 version for Mann-Whitney U test. Correlation analysis was performed by Spearman's rank correlation test using Software STATA/IC-11 (StataCorp, 4905, Lakeway Drive, College station, TX 77845, USA). The P value for significance was set at ≤ 0.05.

4.5 Results

Antibody responses in non-vaccinated but naturally-infected koalas, as well as in vaccinated koalas

Wild koalas sampled from the MBR population were divided into *C. pecorum* PCR-negative and PCR-positive sub-groups following screening of conjunctival and urogenital swabs by our species-specific qPCR. To quantify the presence of *C. pecorum* MOMP and *C. pecorum* EB-specific systemic antibody responses, plasma samples from the naturally-infected and/or vaccinated koalas screened in this study were subjected to *C. pecorum* MOMP and *C. pecorum* EB-specific ELISAs. All five *C. pecorum* PCR-negative animals (not vaccinated) did not have any antibodies to either *C. pecorum* recombinant MOMP protein or to whole chlamydial EBs (Fig. 1; A and B respectively). By comparison, all five *C. pecorum* PCR-positive unvaccinated animals had antibodies that recognised both recombinant MOMP protein (titers ranging from $0.5x10^6$ to $2x10^6$ EPT) as well as whole chlamydial EBs (titers ranging from $0.5x10^3$ to $1x10^3$ EPT; Fig. 1 C and D). Given that we did not know when each animal was initially infected (potentially months to years previously), not surprisingly, the titers remained relatively constant across the 6-month study period.

Following vaccination, all 10 koalas produced antibodies to both rMOMP protein as well as whole *C. pecorum* EBs (Fig. 2. A-D). Interestingly, animals that were PCR-positive at the time of vaccination produced stronger antibody responses than those that were PCR-negative (although there was animal to animal variation). This was particularly evident with the anti-MOMP responses, where four out of five PCR positive animals produced titers of around 3-$4x10^6$ (Fig . 2 C), compared to peak titers for the *C. pecorum* PCR-negative vaccinated

animals of around $1x10^6$, with a single animal at $2x10^6$ (Fig. 2 A). These differences were not observed for the antibody level measured against whole chlamydial EBs (Fig. 2 B)

In vitro neutralisation levels were boosted significantly following vaccination

To assess the function of the antibodies induced in response to natural infection and/or vaccination, *in vitro* neutralisation assays were performed on *C. pecorum*-infected cell culture monolayers. Naturally-infected koalas (*C. pecorum* PCR-positive at time of analysis) were found to have relatively low levels of *in vitro* neutralisation titers ranging from 0.5 to 2.0 fold change with a mean of 1.0 fold change (Fig. 3 C; 1.0 fold represents no change compared to baseline). When these PCR-positive animals received the vaccine, their *in vitro* neutralisation levels were boosted significantly (Mann-Whitney U test was performed to measure the level of significance; though the p value is not statistically significant: $p < 0.056$), increasing to levels of 2.5 to 8.0 fold change, with a mean of 4.8 fold change (Fig. 3 D). In the cohorts that were *C. pecorum* PCR negative and received the vaccine, they also developed *in vitro* neutralising antibodies (1.5 to 3.0 fold change with a mean of 2.7 fold change; Fig. 3 B), although their levels were slightly lower than the *C. pecorum* PCR-positive animals.

Lower plasma antibody titers correlate with higher infectious burden in naturally-infected koalas

In the naturally-infected cohort (which we increased to a total of 14 animals for this aspect), we compared the chlamydial infection level of the animal (as measured by qPCR at the UGT site) with the plasma antibody titers (measured against rMOMP protein). We found a strong and statistically significant correlation ($p < 0.01$) between higher infection load and lower antibody titer (Fig. 4).

Naturally-diseased animals, as well as vaccine cohorts, show unique but distinct peptide antibody response profiles

We used Pepscan methodology to examine the epitope specificity of the antibodies produced in (a) *C. pecorum* PCR-positive animals with and without chlamydial disease, and (b) *C. pecorum* PCR-negative/positive animals following vaccination. We found that diseased koalas (Cougar, Karen and Mango had developed disease at the 6-month time point) (Fig. 5 B1) had plasma antibodies that specifically recognised a unique set of MOMP epitopes 5, 33, 79 and 85. These epitopes were not recognised by plasma from healthy koalas (Fig. 5 A). By comparison, vaccinated koalas produced antibodies that recognised a different unique set of epitopes; 4, 28 and 41 (80% to 90% of koalas tested had responses to these epitopes) and to a lesser level, epitope 42 (40% to 60% of koalas) (Fig. 5 B2).

In vitro neutralisation of post-vaccination plasma is associated with antibodies against specific MOMP epitopes

We observed that the *in vitro* neutralisation titers were highest in animals that were previously infected and then vaccinated (Fig. 3 D). We therefore wanted to know if it was the antibodies to specific epitopes that were responsible for this *in vitro* neutralisation effect. Peptides 4, 28, 41 and 42, shown in the previous experiment to be recognised by vaccinated koalas, were used to adsorb plasma from the infected and/or non-infected plus vaccinated cohort. We then tested the post-adsorption plasma to confirm that we had indeed removed the antibodies specific for these epitopes (Fig. 6 B) and then tested this post-adsorption plasma in an *in vitro* neutralisation assay (Fig. 6 C). The data show that we were successful in removing antibodies against epitopes 4, 28, 41 and 42. Compared to the pre-adsorption plasma which

had an *in vitro* neutralisation fold effect of 4.8 times (Fig. 6 C), the post-adsorption plasma had virtually no *in vitro* neutralising ability (fold change of 1.0) (Fig. 6 C) ($p < 0.001$). This confirms that all, or at least the majority, of the *in vitro* neutralisation was due to antibodies against these specific epitopes.

4.6 Discussion

While our efforts to develop an effective koala chlamydial vaccine are progressing well [11-15, 17], we still do not fully understand which aspects of the koala's immune system are required for an optimal vaccine response. In this project, we analysed the role of antibodies in naturally-infected koalas, as well as koalas that received our prototype vaccine. Vaccine research in the mouse model in particular, has shown that an interferon-gamma T cell response is important [5], but also suggests that neutralising antibodies have a role in protection [18, 19]. In our current study, this analysis revealed a number of new and significant observations that are not only relevant to chlamydial infections in koalas, but also show how the "*C. pecorum*-koala model" might be able to inform human *C. trachomatis* vaccine development. In terms of the latter, a unique aspect of our study was that we vaccinated *C. pecorum*-positive (by PCR) koalas as well as *C. pecorum*-negative koalas. It is not possible to do this in humans, as protocol requires that any *Chlamydia*-positive individuals be immediately treated with antibiotics. Perhaps not surprisingly, in our test koalas, we found higher antibody responses in vaccinated koalas which had a current chlamydial infection. We also observed a different kinetic response, reminiscent of a secondary response, in these animals. While there have been limited studies in human *C. trachomatis* infections, antibodies produced against natural infections generally do not appear to be protective for most individuals [18, 20].

We then examined the functionality of these antibodies by assaying their ability to neutralise *C. pecorum* infections in an *in vitro* neutralisation assay. We first examined the *in vitro* neutralisation ability of antibodies from naturally-infected animals and found that they have very low, to nil antibodies that are capable of neutralisation. This suggests that the antibody response to whole live chlamydiae in a natural infection somehow results in antibodies of specificity that do not negate the infection. By comparison, plasma from vaccinated animals (both animals with current infection but also *Chlamydia*-negative animals) did neutralise *C. pecorum* infections *in vitro*. Somewhat unexpectedly, the plasma from animals which were previously naturally infected (and not protected) but then vaccinated, produced a higher *in vitro* neutralisation effect. We observed two types of antibody responses: for some of the epitopes against which there was a response following natural infection, we observed a boosting of the response to these epitopes following vaccination (eg. epitopes 54 and 87), however, the majority of antibodies produced following vaccination were to new epitopes (eg. epitopes 4, 28, 41, 42).

The concept of "original antigen sin" suggests that a prior exposure to an antigen leads to a sub-optimal second immune response if the strains are too closely related [21]. In the case of *Chlamydia* infections, several previous reports have suggested that original antigen sin may occur when closely related *C. trachomatis* serovars are involved in the priming and challenge infections [21, 22]. In our case of *C. pecorum* infections in koalas, if original antigen sin was occurring, we might have expected that in animals previously infected naturally, our vaccine response may have been limited to boosting only the previously induced antibody types. By comparison, we found that the vaccine induced a new set of novel epitope antibodies (located

elsewhere along the MOMP protein), suggesting that original antigen sin is not of concern in this case. This may have been linked to the fact that most of our epitope responses were to conserved regions of the MOMP protein, although it has also been reported that the use of an adjuvanted vaccine can overcome original antigen sin [23]. Another possible explanation is that the adjuvant Iscomatrix® induced epitope spreading as has been demonstrated in human's immunised with influenza VLPs with Iscomatrix® [24].

Given the promising antibody response that we observed following *C. pecorum* vaccination in our koalas, we wanted to characterize the type of antibodies being produced. We used Pepscan methodology and found that there were two unique epitope profiles. One profile was found in naturally-infected animals, a proportion of which progressed to disease (ie. they were not protected). Recently, we (Waugh et al.-submitted) evaluated the protection provided by vaccinating wild koalas against *Chlamydia*, as measured primarily by the reduction in *Chlamydia* infection load at the ocular and urogenital sites. This study showed that animals with high levels of neutralising antibodies were less likely to progress to disease, which supports a protective role of antibodies. By comparison, vaccinated koalas produced a different, unique profile, against 3-5 key MOMP epitopes. All of these epitopes were in the conserved domain, suggesting that the response should have broad neutralizing ability across multiple *C. pecorum* strains. While it has previously been assumed that antibodies must bind to externally presented epitopes to enable neutralisation [25], recent reports have shown that antibodies, via FcRn mediation, can internalize and neutralise virus [26] and intracellular bacteria such as *Chlamydia*, within the cytoplasm of epithelial cells [27]. Antibodies to internal proteins such as NrdB have also been shown previously to facilitate the neutralisation process [28]. To confirm the specificity of these epitope responses, we used the individual

peptides to adsorb the specific antibodies out of these groups and then re-tested their *in vitro* neutralising ability. This clearly demonstrated that the *in vitro* neutralisation ability was due to the novel peptide responses following vaccination.

While MOMP is known to be highly antigenic, [29] it is also highly variable across koala *C. pecorum* strains [30]. This means that for a vaccine to be broadly protective under field conditions, it may need to contain a range of MOMP variants (we used three full length MOMP proteins in our vaccine cohort). A defined epitope vaccine would be an ideal approach to overcoming this problem and could also avoid any deleterious epitopes. Recent studies found that systemic and mucosal immune responses could be generated with the induction of a peptide-based vaccine for *C. trachomatis* infection [31], which highlights the promising aspect of these investigations for the koala. In addition to antibodies, a strong and specific T cell response is also necessary for an effective vaccine [32]. It will therefore be necessary in future to identify the key T cell epitopes in koala *C. pecorum* MOMP.

Our findings provide novel information in relation to the antibody responses in naturally-infected koalas as well as koalas following vaccination, which should significantly inform future vaccine development. Firstly, even though naturally-infected koalas produce an antibody response, it is of relatively low total titer, does not neutralise, and is apparently non-protective against disease progression. Secondly, the response is to epitopes in the constant domains of MOMP, although why the host/parasite has "directed" the response to these epitopes (non-protective) is not clear. Finally, additional antibodies to epitopes not present in naturally-infected koalas can be induced following vaccination, and these additional epitopes are able to neutralise whole EBs in a live assay.

4.7 Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

4.8 References

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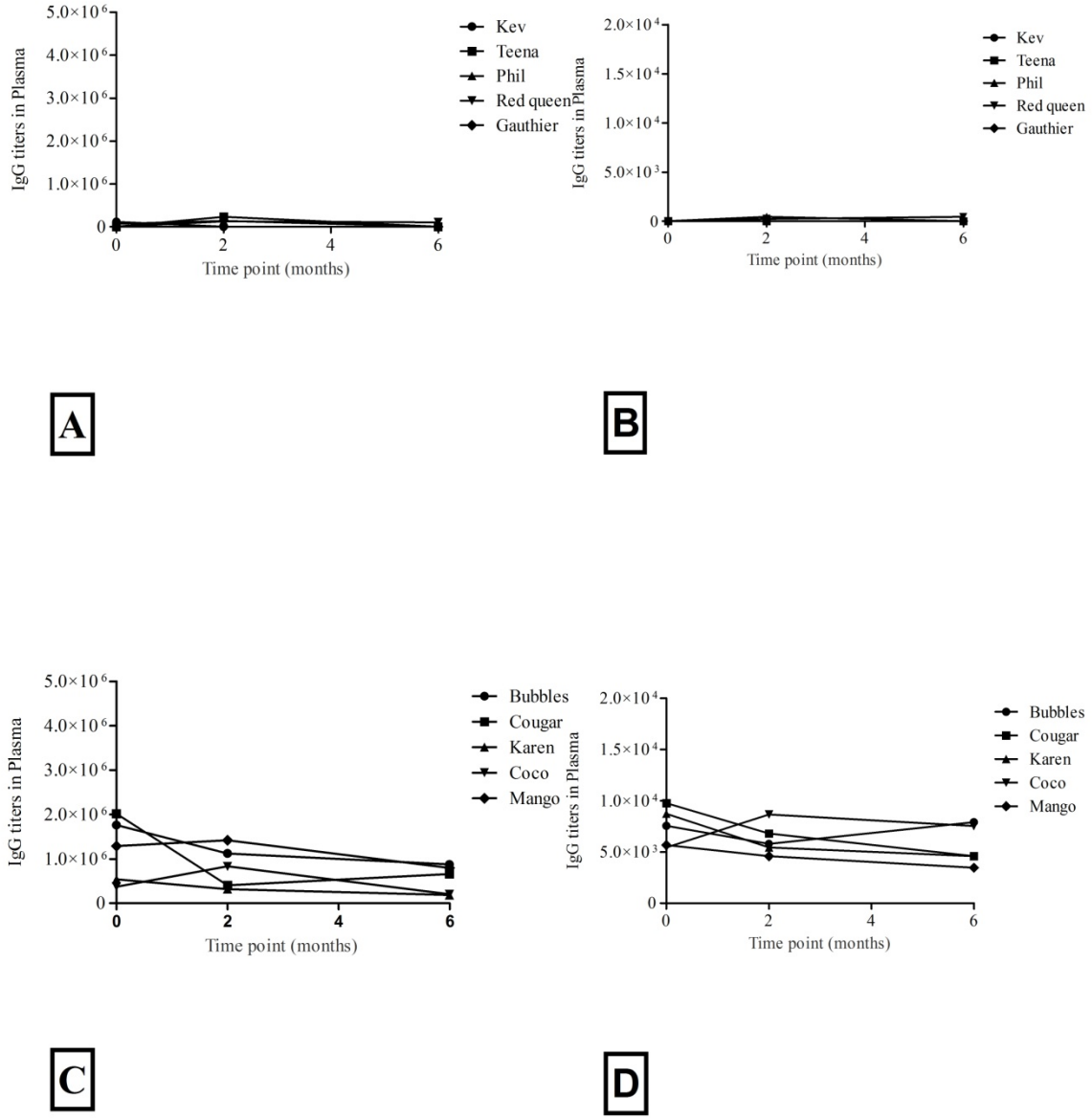


Fig. 1. The circulatory antibody titers (IgG) against rMOMP (A and C) and EBs (B and D) in *Chlamydia*-negative (Group A and B) and naturally-infected animals (Group C and D) at the initial time point. Five animals were included in each cohort.

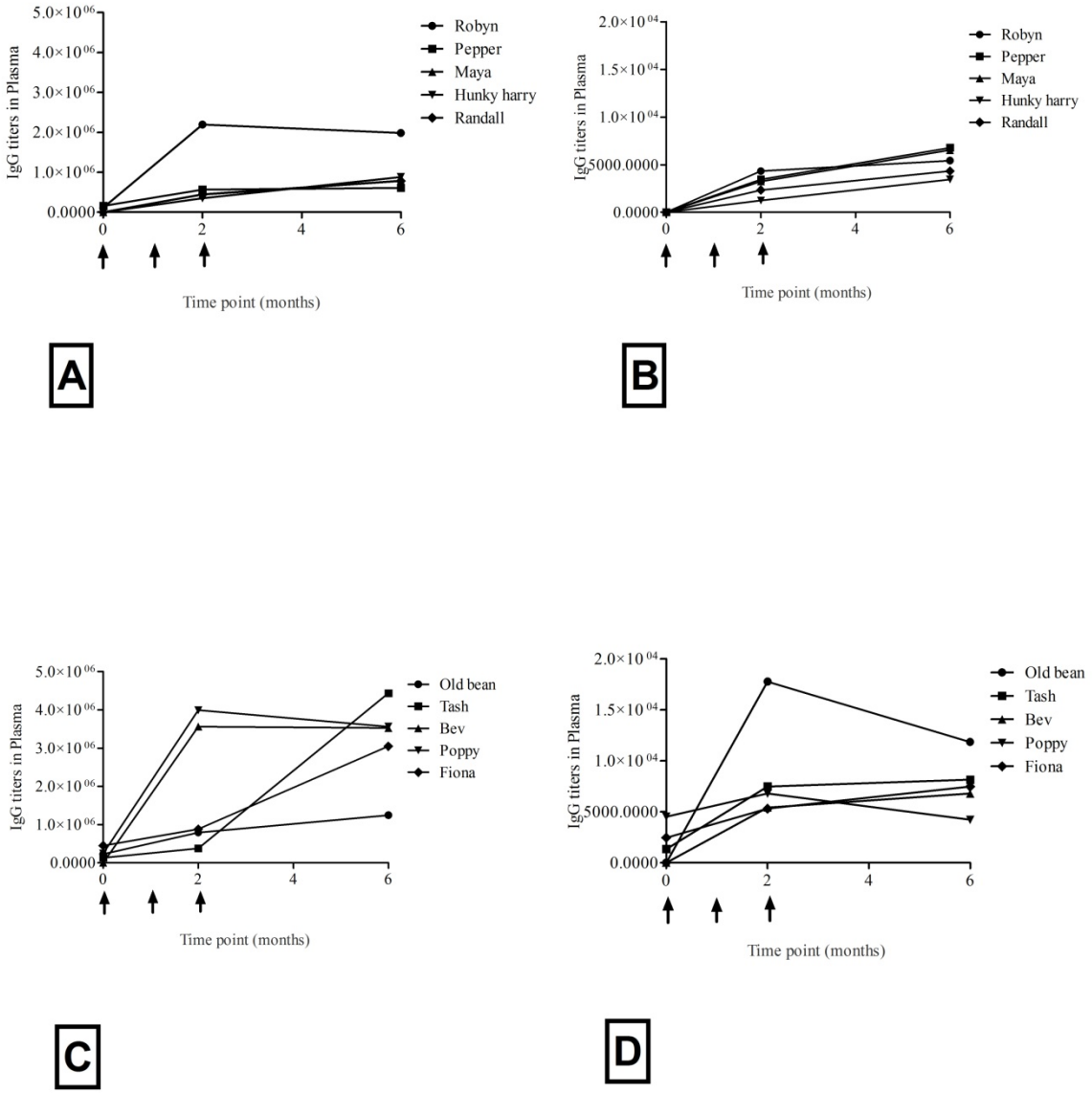


Fig. 2. Antibody titers (IgG) against rMOMP (A and C) and EBs (B and D) in plasma from *Chlamydia*-negative animals (Groups A and B) or naturally-infected animals (Group C and D) at the initial time point. Both groups were vaccinated three times at 1-month intervals. Five animals were included in each cohort. Arrows indicate the vaccination time points.

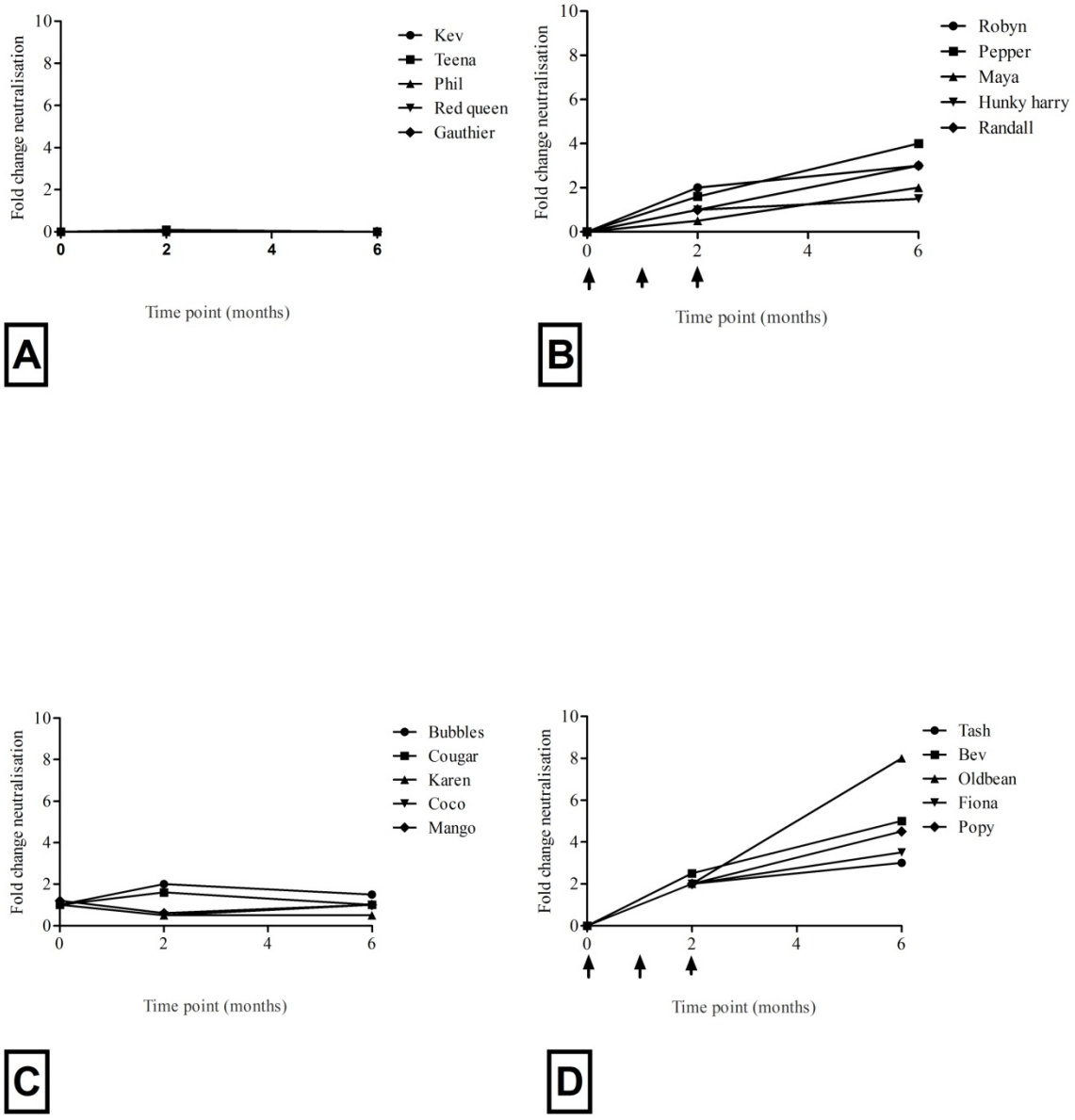


Fig. 3. Fold change in neutralising antibodies in different cohorts of koalas. *Chlamydia*-negative animals without (3A) or following vaccination (3B) and *Chlamydia*-infected koalas without (3C) and with vaccination (3D) are shown. Arrows indicate the vaccination time points. All samples were diluted 1:10 in media and results are expressed as fold change neutralisation of immune samples compared to those pre-immune samples. The level of

significance was measured as *p - 0.01-0.05, **p - 0.001-0.01, ***p<0.001. Mann-Whitney U test was performed to measure the level of significance (p <0.056).

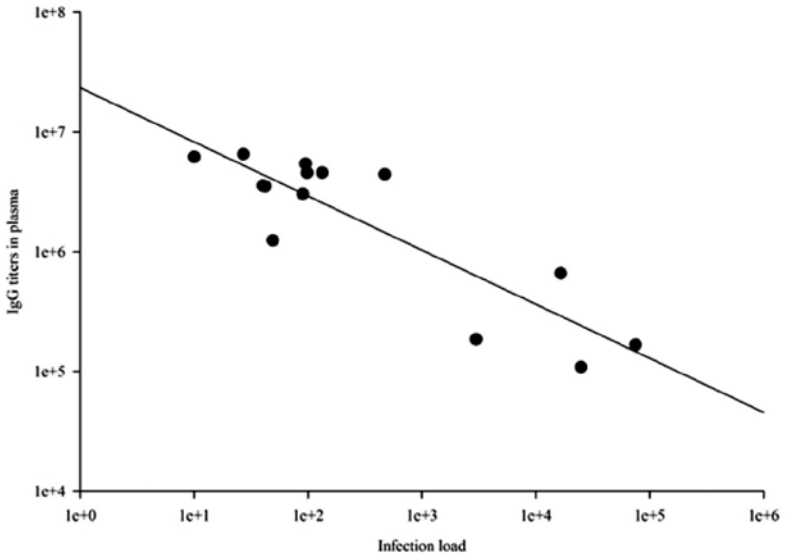


Fig. 4. The correlation of bacterial burden measured by *C. pecorum*-specific qPCR and antibody titers in koalas with current infection (n =14) at the initial enrolment. The infection loads were measured at the UGT. The Spearman's correlation coefficients were performed to measure the relationship between IgG and infection load. The level of significance was measured as *p - 0.01-0.05, **p - 0.001-0.01, ***p<0.001

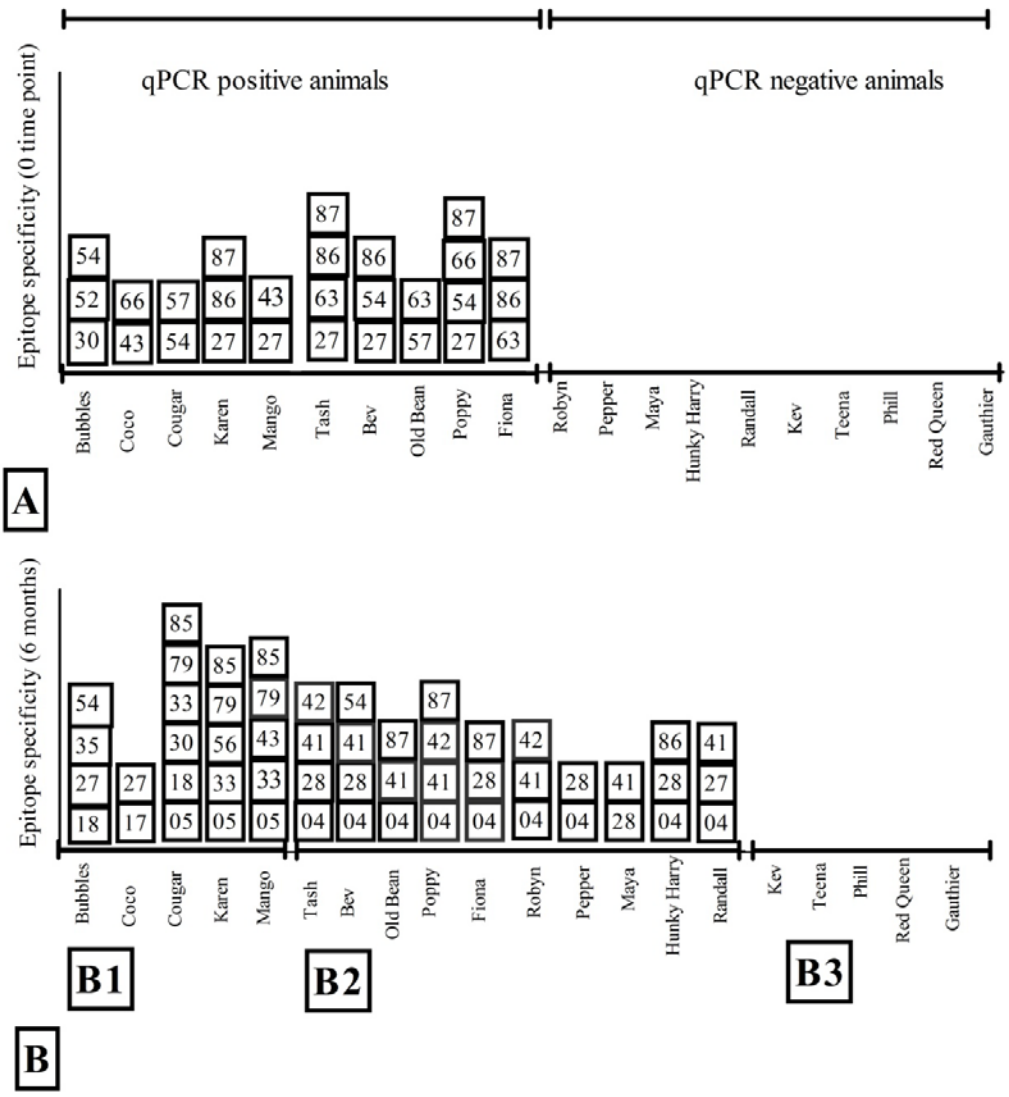


Fig. 5. Specific epitopes detected in koalas with current *C. pecorum* infection and/or following vaccination. The antibody-specific epitopes detected in infected and non-infected animals is shown at 0 months (5A). At the 6-month time point, antibody-specific epitopes (5 B) were measured in animals with and without vaccination. (B1= Animals naturally infected: Bubbles, Coco and progressing to disease: Cougar, Karen and Mango; B2= naturally infected following vaccination for Tash, Bev, Old Bean, Poppy and Fiona and non-infected animals following vaccination for Robyn, Pepper, Maya, Hunky Harry and Randall; B3= *Chlamydia*-negative animals without vaccination).

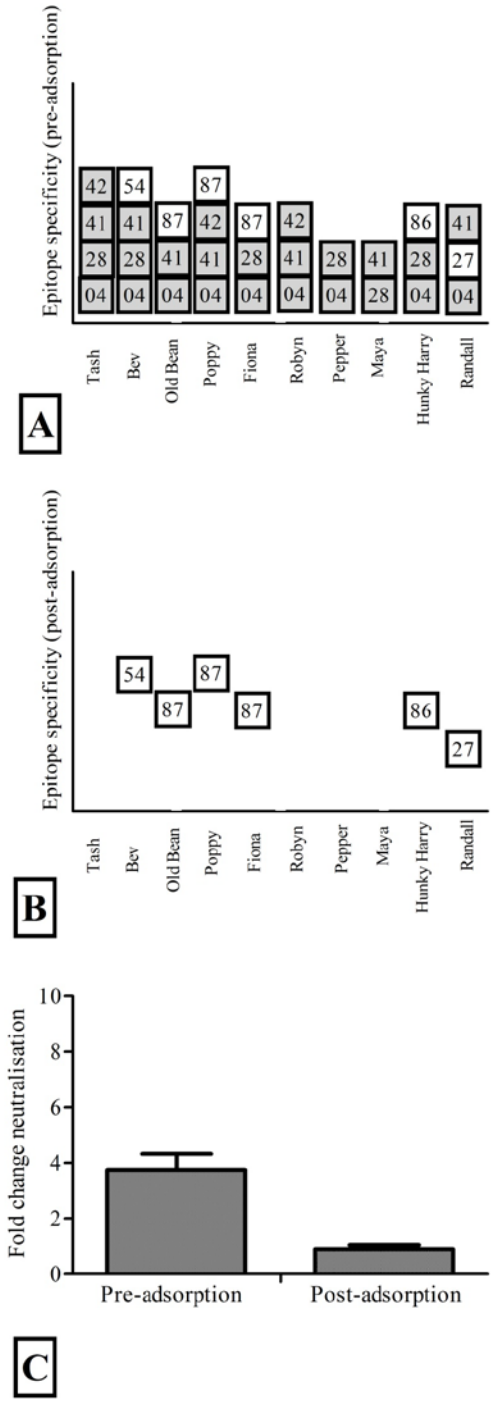
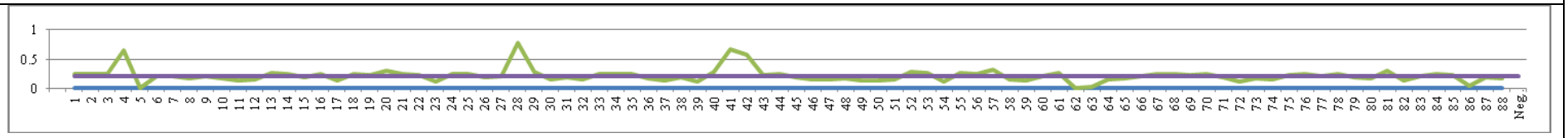
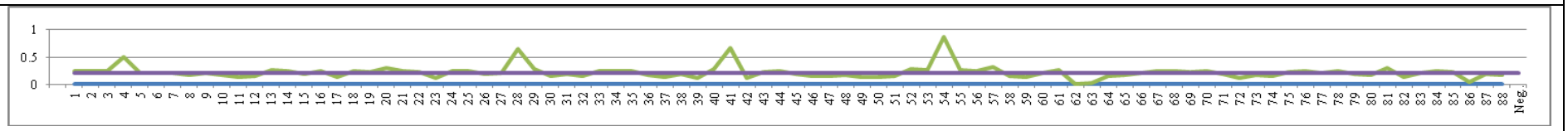
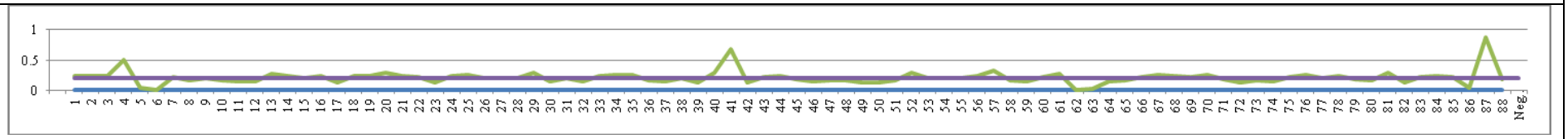
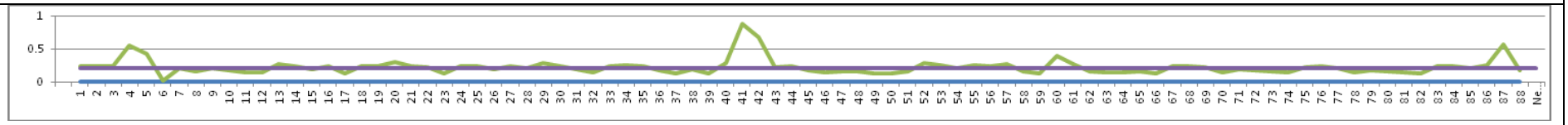
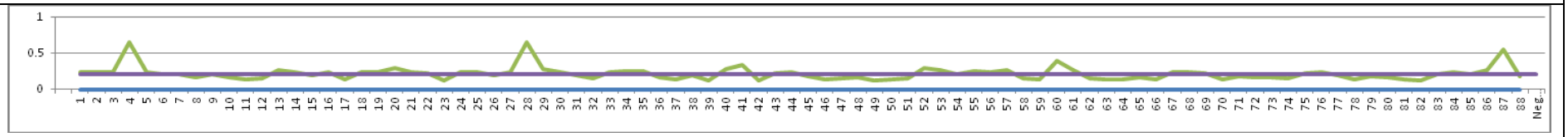


Fig. 6. Epitopes detected antibodies and *C. pecorum*-specific neutralisation in vaccinated cohort. The grey boxes indicate the vaccine induced unique epitopes response. The epitope response in plasma before (Fig 6 A) and after (Fig 6 B) adsorption out of the positive

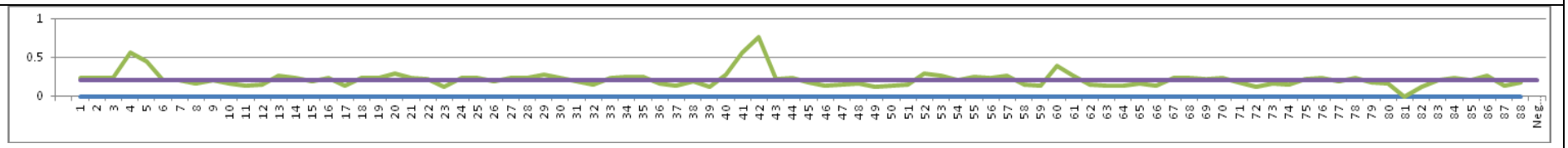
epitopes (4, 28, 41 and 42) is shown. All samples were diluted 1:10 and results are expressed as fold change neutralisation of immune samples compared to those pre-immune samples. The fold change neutralisation as pre and post-adsorption (Fig 6 C) cohorts. The level of significance was measured as *p - 0.01-0.05, **p - 0.001-0.01, ***p<0.001. Significant reduction of neutralising antibodies in post-adsorption plasma (p < 001). Results are expressed as the mean ± SD of 10 animals per group. Error bars represent SD.

Epitopes mapping for *C. pecorum* infected koalas following vaccination

Tash	
Bev	
Old Bean	
Poppy	
Fiona	
	

Epitopes mapping for koalas having no chlamydial infection following vaccination

Robyn



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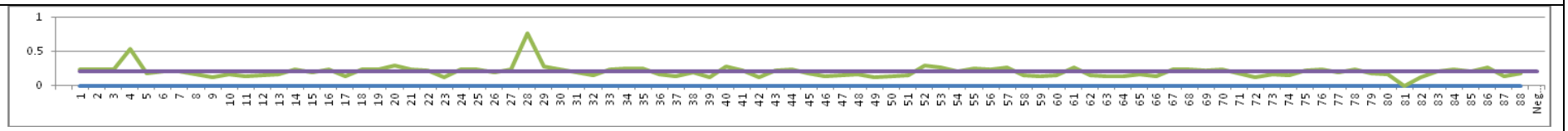

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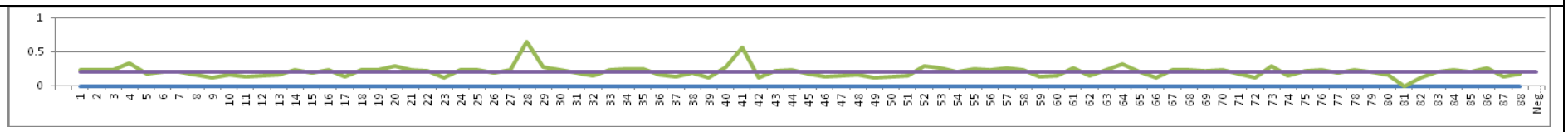

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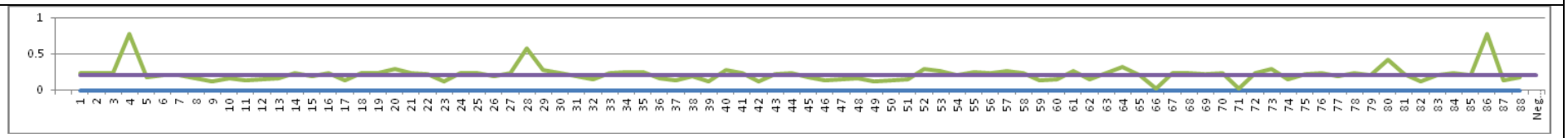

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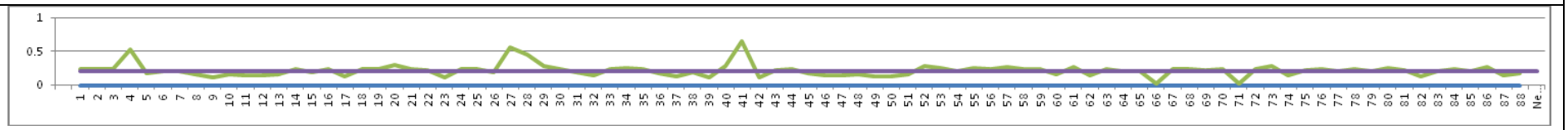
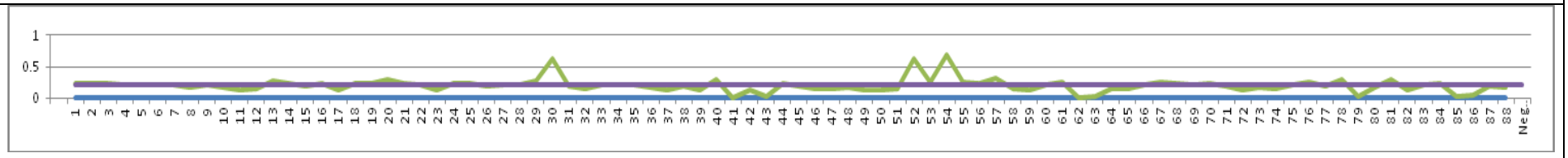
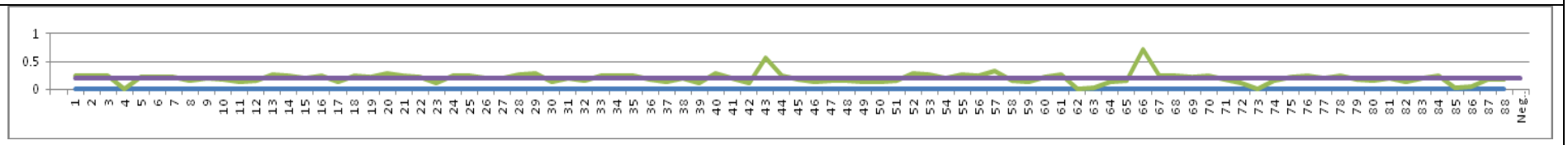
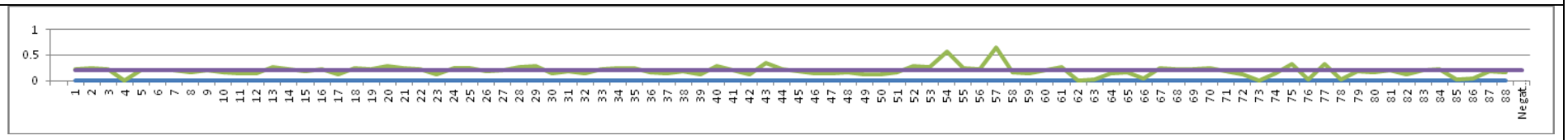
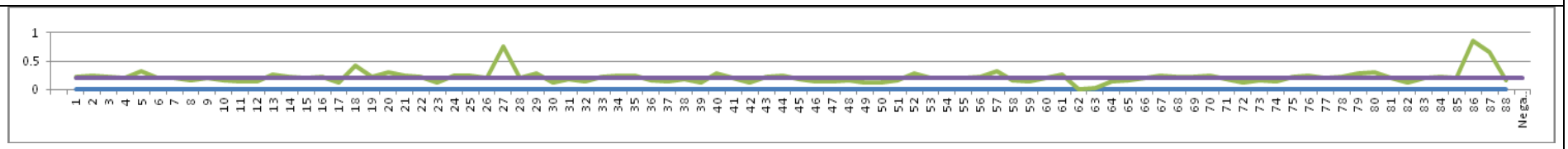
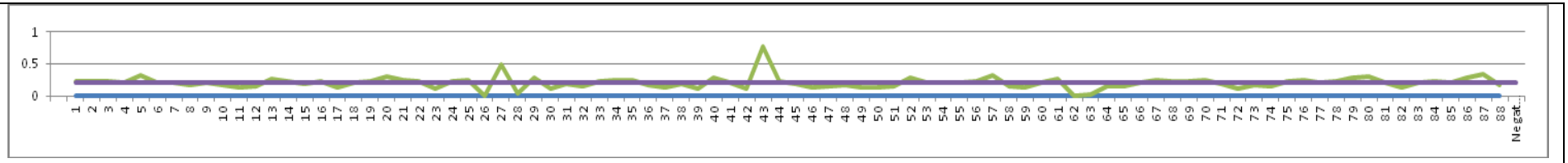
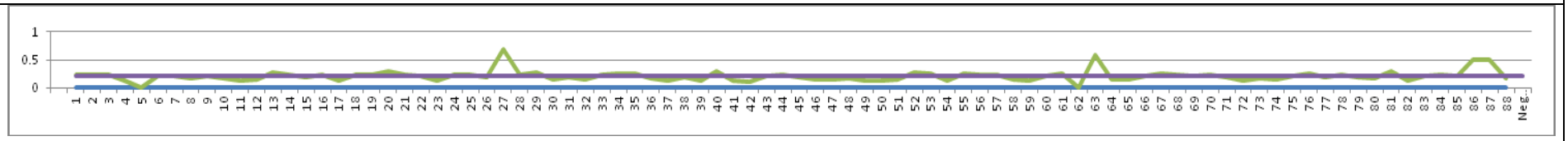
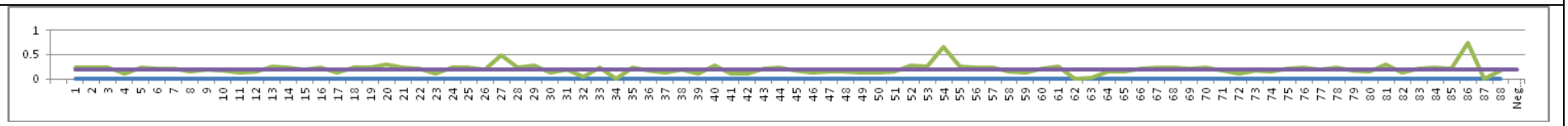
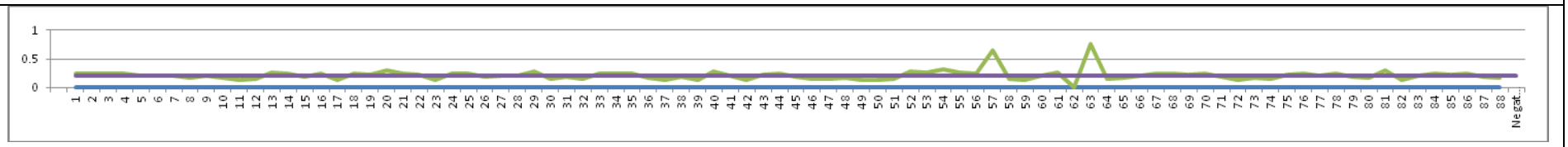
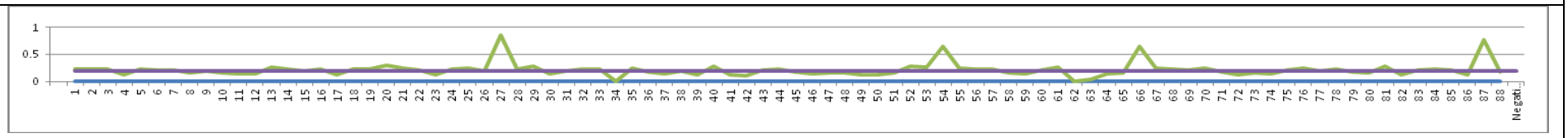


Epitopes mapping in koalas with *C. pecorum* infection	
Bubbles	
Coco	
Cougar	
Karen	

Mango	
	
Tash	
Bev	
Old Bean	
Poppy	

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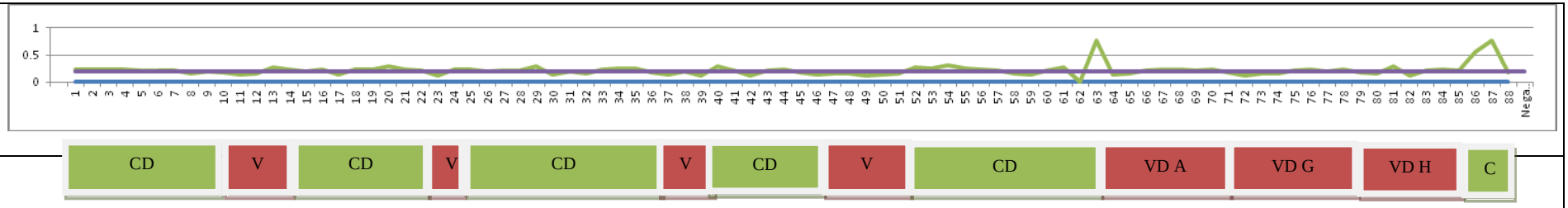
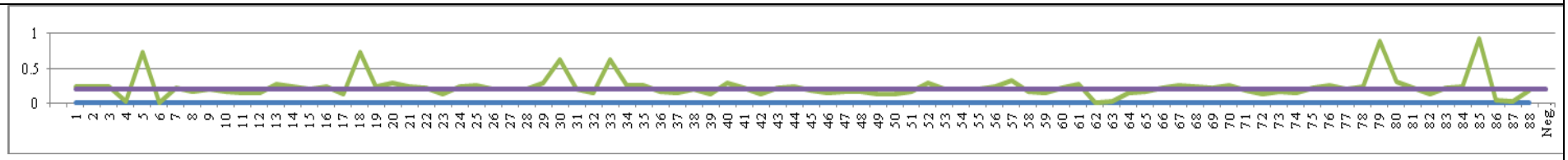


Epitopes mapping in koalas with clinical *C. pecorum* infection

Cougar



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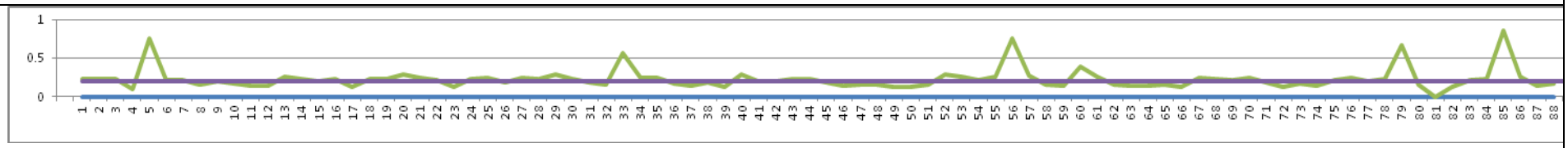

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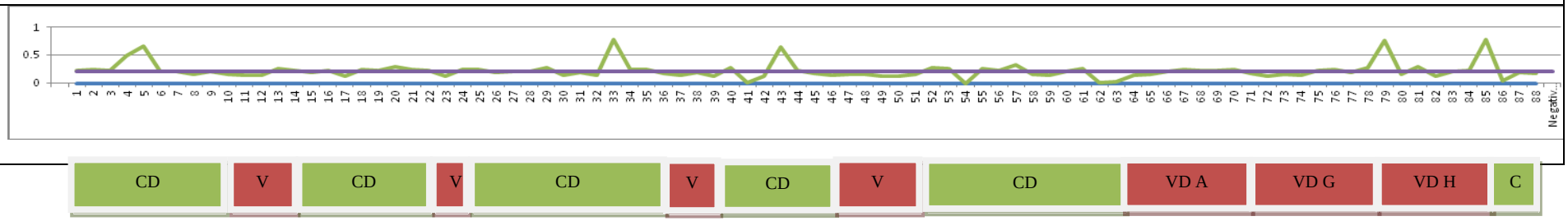

<u>Chapter 5:</u> A prototype recombinant-protein based *Chlamydia pecorum* vaccine results in reduced chlamydial burden and less clinical disease in free-ranging koalas (*Phascolarctos cinereus*)

5.1 Statement of contribution of co-authors for thesis by published paper

The authors listed below have certified that:

1. They meet the criteria for authorship in that they have participated in the conception, or execution, or interpretation, of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to (a) granting bodies, and (b) the editor or publisher of PloS one and
5. They agree to the use of the publication in the student's thesis and its publication on the QUT ePrints database consistent with any limitations set by publisher requirements.

In the case of this chapter:

Waugh, C, **Khan, S.A.,** Carver, S, Hanger, J., Loader, J., Polkinghorne, A., Beagley, K., and Timms, P. (2016). A Prototype Recombinant-Protein Based *Chlamydia pecorum* Vaccine Results in Reduced Chlamydial Burden and Less Clinical Disease in Free-Ranging Koalas (*Phascolarctos cinereus*). PLoS one; 11(1): e0146934.

Contributor	Statement
Courtney Waugh Signature Date	Contributed to the project design and over all implementation, conducted the majority of the laboratory work in the project, conducted the analysis and interpretation of the data and wrote the manuscript
Shahneaz Ali Khan	Contribution to the laboratory work for the ELISA component as well as analysis and manuscript editing
Scott carver	Contributed the statistical analysis and critically reading the manuscript and editing
Jon Hanger	Veterinary expertise and contributed to thorough clinical examination and sampling of koalas in this large field study.
Jo Loader	Veterinary expertise and contributed to the sampling of koalas and involved in the collaboration and approval of this study
Adam Polkinghorne	Contributed to critical reading , editing of the manuscript
Kenneth Beagley	Contributed to the critical reading and editing of the manuscript
Peter Timms	Designed the initial research plan; involved in experimental planning , design and securing funding; major contributor in collaborating with the industry partners; critical reading, editing and approved the manuscript

Principal Supervisor Confirmation

I have sighted email or other correspondence from all co-authors confirming their certifying authorship.

PETER TIMMS — Name

Signature

3/5/2016 — Date

5.2 Abstract

Diseases associated with *Chlamydia pecorum* infection are a major cause of decline in koala populations in Australia. While koalas in care can generally be treated, a vaccine is considered the only option to effectively reduce the threat of infection and disease at the population level. In the current study, we vaccinated 30 free-ranging koalas with a prototype *Chlamydia pecorum* vaccine consisting of a recombinant chlamydial MOMP adjuvanted with an immune stimulating complex. An additional cohort of 30 animals did not receive any vaccine and acted as comparison controls. Animals accepted into this study were either uninfected (*Chlamydia* PCR negative) at time of initial vaccination, or infected (*C. pecorum* positive) at either urogenital (UGT) and/or ocular sites (Oc), but with no clinical signs of chlamydial disease. All koalas were vaccinated / sampled and then re-released into their natural habitat before re-capturing and re-sampling at 6 and 12 months. All vaccinated koalas produced a strong immune response to the vaccine, as indicated by high titres of specific plasma antibodies. The incidence of new infections in vaccinated koalas over the 12-month period post-vaccination was slightly less than koalas in the control group, however, this was not statistically significant. Importantly though, the vaccine was able to significantly reduce the infectious load in animals that were *Chlamydia* positive at the time of vaccination. This effect was evident at both the Oc and UGT sites and was stronger at 6 months than at 12 months post-vaccination. Finally, the vaccine was also able to reduce the number of animals that progressed to disease during the 12-month period. While the sample sizes were small (statistically speaking), results were nonetheless striking. This study highlights the potential for successful development of a *Chlamydia* vaccine for koalas in a wild setting.

Keywords *Chlamydia*; Vaccine; Major Outer Membrane Proteins; Therapeutic vaccine; Koala; *Phascolarctos cinereus*

5.3 Introduction

Infections by the intracellular bacterium *Chlamydia pecorum* contribute to significant morbidity and mortality in the koala (*Phascolarctos cinereus*). Disease progression can include kerato-conjunctivitis, cystitis, reproductive disease/sterility and blindness; the progression of which, in severe cases, can cause death. An antibiotic treatment regime is currently recommended for mild infections [1], however for koalas affected by severe chlamydial disease, antibiotics alone are not sufficient to cure the clinical signs [1].

In recognition that a reduction in disease may have a positive effect in the conservation of koalas [2, 3], our group has been leading the development of a prototype *C. pecorum* vaccine [4-9]. Based on studies which have shown efficacy in animal models (reviewed in Farris and Morrison [10]), the primary component of the *C. pecorum* vaccine has been the recombinant proteins derived from the chlamydial Major Outer Membrane Protein (rMOMP). rMOMP is highly immunogenic in humans and animals and has been studied in detail as a vaccine candidate. In the initial studies utilizing this vaccine antigen adjuvanted with an immune stimulating complex, we have shown that this prototype chlamydial vaccine (i) induces long-lasting specific humoral and cell-mediated immune responses in vaccinated koalas [9]; (ii) induces an immune response that can recognize genetically distinct *C. pecorum* strains, a capability that natural infection does not appear to have [6]; (iii) induces the production of

specific antibodies that are effective in neutralizing *C. pecorum in vitro* [9]; and (iv) does not have any apparent deleterious effects on the health of *Chlamydia*-free koalas or koalas with current chlamydial infection and/or disease [8, 11].

In the absence of an established infection challenge model for the koala, further understanding of the efficacy of the vaccine for reducing the risk and impact of chlamydial infection at both the individual and population level is limited. In the current study, we assessed the health outcomes of a cohort of 60 koalas, including 30 animals vaccinated with the prototype *Chlamydia* vaccine within one free-ranging population in South-East Queensland (SEQ), Australia. Vaccinated and control cohorts of animals were then released, monitored for a period of 12 months, and recaptured periodically to compare a range of health parameters between the two groups.

5.4 Materials and Methods

Chlamydia pecorum MOMP recombinant preparation

Purified *C. pecorum* MOMP from three koala *C. pecorum* genotypes (A, F and G) were used as previously described by Kollipara et al. [7].

Animals and Immunizations

Animals included in the study (n = 60) were part of a larger population-wide study by the Queensland Government Department of Transport and Main Roads (as part of the Moreton Bay Rail Link project), conducted between 2012 and 2015 in the Moreton Bay Region,

Queensland, Australia. Criteria for inclusion into the study were animals of breeding age (>1 year) of either sex, with no clinical signs of chlamydial disease, as assessed during the initial capture event by qualified wildlife veterinarians. Animals were randomly assigned to either the vaccinated or control (non-vaccinated) group at initial capture. The vaccinated group (n = 30) received a three-dose regime of the vaccine via the sub-cutaneous route, given at one-month intervals, consisting of the three rMOMP proteins as the antigens (50µg each of MOMP-G, MOMP-A, and MOMP-F) and an Immunostimulating complex adjuvant (50µg , ISC, Zoetis Australia [4]). Following a detailed veterinary health assessment, animals were released with a radio collar or anklet for tracking (Sirtrack). Animals were re-captured at 1 month, 2 months, 6 months, and 12 months for the purpose of (i) additional vaccinations for the vaccine cohort animals only (1 month and 2 months) or (ii) detailed health checks and sampling (2, 6 and 12 months). While 30 animals were originally recruited into each group, unfortunately, only 23 vaccinated and 27 control koalas could be resampled at the six month time point due to animal losses associated with misadventure (e.g. predation, trauma, koala movements outside of study area, or disease). At 12 months, again, further losses had occurred and numbers were considerably reduced in each cohort to 15 vaccinated and 14 control koalas.

All procedures were approved by the University of the Sunshine Coast (USC) Animal Ethics Committee (Animal ethics number AN/A/13/80) and by the Queensland Government (Scientific Purposes Permit, WISP11532912). The trial was performed under the Australian Pesticides and Veterinary Medicines Authority Permit PER 7250.

Health assessments and sampling

Veterinary assessments and sampling, while under a short period of anesthesia, were conducted on each animal at 0, 2, 6, and 12 month time-points following their initial capture and veterinary examination. Ultrasound examination of the kidneys, ureters, urinary bladder and the reproductive tract allowed for identification of urogenital tract diseases including cystitis and reproductive-tract cysts in female koalas. Urinalysis was utilized to detect possible kidney or urinary tract disorders, such as cystitis, which is also associated with *Chlamydia*. Chlamydial disease scores were assessed according to the disease scoring criteria outlined in detail in Wan et al. [12]. For the purposes of this study, one set of conjunctival/ocular (Oc) and urogenital (UGT) swabs were collected for *Chlamydia* load determination and a blood sample of up to 5mL was collected from the cephalic vein. This was used for preparation of haematology smears and separation of plasma and serum by centrifugation.

Chlamydia-specific IgG plasma response

IgG response was analysed via enzyme-linked immunosorbent assays (ELISAs). ELISAs were performed on plasma samples at 0 and 6 months as per Khan et al. [5], and served as a control to demonstrate that vaccinated koalas produced a specific immune response to the vaccine antigens as previously shown [5

C. pecorum quantification

Swab samples were stored at -20°C until the DNA was extracted as described by Devereaux et al. [13]. The extracted samples were screened for the presence of *C. pecorum* using a diagnostic quantitative real-time PCR (RT-PCR) targeting a 204 bp fragment of the

chlamydial 16S rRNA gene. Assays were as described in Marsh et al. [14] except for the PCR mixture containing 1× QuantiTect SYBR Green PCR Master Mix (Qiagen) and 10 μM primers [14] made up to a final volume of 15 μl with PCR-grade water, as well as an increased initial denaturation to 15 mins at 94°C. All reactions were carried out on a Rotor-Gene Q 5-plex HRM platform (Qiagen).

Statistical analysis

Significant differences between 0 and 6 month IgG antibody titres were evaluated with a Wilcoxon signed-rank test. To evaluate how *C. pecorum* infection prevalence and loads differed among vaccinated versus control koalas, Chi-square contingency table analyses were used to compare the changes in *C. pecorum* load over time (0 vs. 6 and 0 vs. 12 months), with changes categorized into bands as either, decreasing, stable or increasing (ΔqPCR $\leq$ -100, -99 – 99, and $\geq$ 100 copies/μL respectively). These categories were chosen because small variations of up to 100 copies/μL in qPCR can occur across assays. We conducted this analysis on the raw numbers of koalas within each group (which we considered a conservative analysis, given our sample size), and on the percentage of koalas in each group (which we considered a more sensitive approach, given our sample size). We chose these conservative and sensitive approaches because, though our results are striking, the numerical effect of koala mortalities in the field inflated the Type-II statistical error, limiting detection of statistical significance based on raw data alone. Where appropriate we used Markov chain Monte Carlo simulations to overcome Chi-square statistical issues associated with expected values < 5. Analyses were conducted on both Oc and UGT infections (see Results). All analyses were conducted using Rv3.0.2 (www.r-project.org).

5.5 Results

Vaccine safety data

All vaccinated animals were monitored for up to 24 hours post-vaccination and given a thorough veterinary health check at 2 months and thereafter at their regular 6-monthly capture and sampling events. There were no short or longer-term adverse events reported due to administration of the vaccine in any of the animals.

Immune response to vaccination

We used our *Chlamydia* ELISA to determine plasma IgG antibody levels both (i) at 0 months and (ii) 6 months post vaccination in the vaccine group (n = 23). We found that the average antibody titre at 6 months post-vaccination in PCR negative and PCR positive animals was significantly greater than at 0 months (Figure 1; PCR negative $p = 0.002$; PCR positive $p < 0.001$; Supp Info 1) indicating that we had successfully induced a vaccination-specific immune response.

Chlamydia prevalence

Overall, the 60 animals initially included in our study had a *C. pecorum* prevalence of 54% at time of capture (as defined by *C. pecorum* species-specific PCR). After recruitment into the trial, the koalas were assigned into groups consisting of: (i) animals with a current infection, as defined by being *C. pecorum* PCR positive, at the Oc site (Vaccinated: n = 10; Control: n = 6); (ii) *C. pecorum* PCR positive animals at the UGT site (Vaccinated: n = 8; Control: n = 13); and (iii) animals that were *C. pecorum* PCR negative at either site (Vaccinated: n = 16; Control n = 21). Some animals (Vaccinated: n = 7; Control n = 6) were necessarily included in both groups due to the occurrence of *C. pecorum* positivity at both sites. At 12

months, the number of animals remaining in each group decreased (mortality among wild koalas) to: (i) *C. pecorum* PCR positive animals at the Oc site (Vaccinated: n = 7; Control: n = 4); (ii) *C. pecorum* PCR positive animals at the UGT site (Vaccinated: n = 5; Control: n = 6; and (iii) *C. pecorum* PCR negative animals (Vaccinated: n = 11; Control n = 10).

Rate of new *C. pecorum* infections in vaccinated koalas compared to unvaccinated controls

For this analysis we utilized the animals that were *C. pecorum* negative at 0 months and able to be recaptured at 12 months. In the control group, the 12 month incidence rate at the UGT site was 25% (two new infections in the 8 animals in this group), and 20% (2/10) at the Oc site. By comparison, the vaccinated animals had a slightly lower 12 month incidence rate of 20% (2/10) and 12% (1/8) at the UGT and Oc sites respectively. While the incidence rate in the vaccinated group was lower, the group size was small and hence the difference was not statistically significant (raw data $X^2 = 0.392$, $p > 0.999$; percentage differences $X^2 = 2.381$, $p = 0.176$).

Changes in *Chlamydia* load following vaccination

For animals that were infected (PCR positive) at the time of recruitment, we measured their *Chlamydia* load by quantitative-PCR (qPCR) at 0, 6, and 12 months to evaluate the effect the vaccine had on the level of chlamydial shedding (Table 1). For the purposes of analysis, we grouped the animals into three categories, based on their PCR load change, whether the load decreased, stayed stable, or increased (ΔqPCR $\leq$ -100, -99 – 99, and $\geq$ 100 copies/µL respectively).

At 6 months post-vaccination, animals in the vaccine group were significantly more likely to decrease or stabilize their chlamydial load, whereas animals in the control group were significantly more likely to increase their load (Table 1). This effect was observed as a near significant trend ($p > 0.01$) using the conservative (raw) data and a significant effect based on the more sensitive (%) data. For example, at the ocular site 90% (9/10) of vaccinated animals decreased or stabilized their load, compared to the control group where only 33% (2/6) had decreasing or stabilizing loads (Table 1). Similarly, at the UGT site, 100% (8/8) of animals in the vaccinated group had decreasing or stabilizing loads compared to 69% (9/13) in the control group (Table 1).

At 12 months, the positive vaccine effect was maintained at the UGT site with 100% (5/5) of vaccinated animals showing a decrease in chlamydial load compared to 83% (5/6) in the control group (Table 1). We are cautious about drawing conclusions on the statistical significance of this owing to the difference of only a single individual. However importantly, throughout the entire study, not one animal in the vaccine group showed an increase at the UGT site. At the Oc site at 12 months, 100% (7/7) of vaccinated animals also decreased or stabilized their chlamydial load; although a similar trend (100% [4/4] decrease) was seen in the control group (Table 1). Again, we are highly cautious about interpreting the statistical significance of this based on the sample size. Overall, smaller sample sizes of koalas, owing to field mortalities, cause us to be cautious about statistical interpretation of results at 12 months.

Progression to chlamydial disease

To investigate the impact that vaccination had on the progression of chlamydial disease, we compared the presence and absence of disease in vaccinated and control animals. Over the

12 months of the study, only 1 of 23 (4% of koalas) vaccinated animals developed clinical signs of chlamydial disease, whereas 4 of 27 (14.8%) control animals developed clinical disease over the same time period. Based on percentage differences, the control and vaccinated groups were significantly dissimilar (X^2 = 7.037, p = 0.013), but the same result could not be observed in the raw data (X^2 = 1.512, p = 0.363) owing to the sample size. The one vaccinated animal developed mild, sub-acute, chronic cystitis, was treated in care with the standard chloramphenicol dosage and released as healthy. Three of the four animals that developed disease in the control group developed cystitis and were treated; the final animal developed severe and extensive reproductive disease as well as severe chronic cystitis, and was euthanized.

5.6 Discussion

We have, for the first time, examined the effect of a rMOMP based anti-chlamydial vaccine on chlamydial infection risk and outcome in free-ranging koalas. The vaccine induced a significant immune response in wild-caught koalas. The incidence of new *C. pecorum* infections was lower at both anatomical sites in vaccinated animals, despite not being statistically significant. Importantly, we also found that vaccinated koalas were more likely to have stable or decreasing *C. pecorum* PCR loads, and were also less likely to increase their chlamydial burdens at 6 months post-vaccination at both anatomical sites. At 12 months, this positive effect could still be observed in the vaccinated cohort, with no animals increasing their chlamydial loads at either anatomical site. However, we caution the low number of koalas at this time point made statistical inference unreliable. Lastly, we showed a positive effect for protecting against progression to disease in vaccinated animals.

Therapeutic vaccines are a promising new approach to enhance immunogenicity, and reduce viral and bacterial load in infected humans and animals [15]. Due to the difficulties associated with antibiotic treatment in the koala, a therapeutic vaccine may provide an important alternative to reduce infection. While antibiotics are curative in many cases of chlamydial disease, the therapeutic course is relatively long and labour intensive, often precluding its efficacy for the treatment of koala outside of the clinic. Therefore, a therapeutic vaccine provides a more practical solution for disease management at a non-captive population level, particularly if a single-dose vaccine were to be developed. In our current study, the positive therapeutic effect seen at both anatomical sites in the koala is a promising result for the development of a therapeutic chlamydial vaccine for this species. The loss of meaningful statistical inference at 12 months due to severe field mortalities is disappointing, masking our ability to confidently detect an effect at this time interval. This effect seems largely skewed by the four animals in the control group reducing their chlamydial ocular burden. When followed longitudinally up to two years, two of these four animals developed clinical signs of disease, whereas none of the vaccinated animals in that cohort developed any clinical signs (unpublished data).

While we did not observe a significant improvement in the risk of new infections in the vaccinated koalas, it was interesting to note nevertheless that asymptomatically infected control animals were more likely to advance to disease than asymptomatically infected vaccinated animals. Promisingly, over 12 months, only one vaccinated animal developed new disease symptoms (cystitis), whereas 4 animals in the non-vaccinated cohort succumbed to disease (cystitis n = 3 and reproductive disease n = 1). While beyond the scope of this manuscript, it is also promising to observe that available longitudinal data for the remaining

animals at 18-24 months suggests that two (of 7; 28.6%) additional control animals contracted cystitis, whereas none of the vaccinated animals has yet succumbed to disease (0/6; 0%).

To conclude, the first field trial to date of this prototype koala chlamydial vaccine suggests that vaccinated *Chlamydia*-infected koalas have an improved infection outcome – an outcome that highlights the potential for the development of a therapeutic vaccination schedule for this species. This is especially promising given the small sample sizes, and the natural variability of an outbred population. In the koala, the main goal for population management from an ecological standpoint is maintaining health and young animal recruitment. Therefore, if a vaccine is able to lower or prevent increases of infection load, as well as to decrease the progression to disease, than this will have positive effects on population health and fecundity and may be an important tool in the management and conservation of the koala.

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Conflicts of interest: none.

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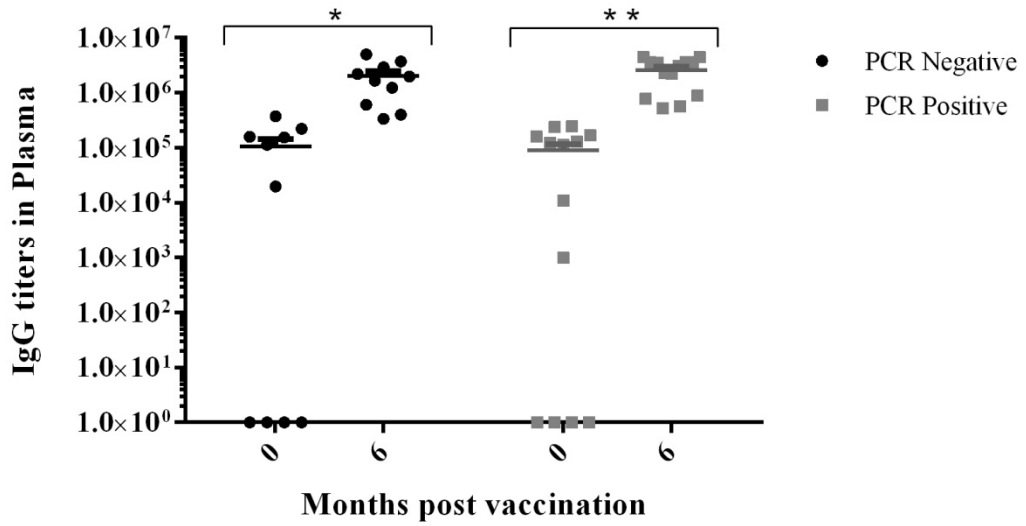

Fig. 1. Antibody (IgG) titre response in vaccinated animals at 0 months (pre-vaccination) and 6 months post-vaccination (n = 23): Mean and standard error of IgG titre levels. $P < 0.05$ *; $P < 0.001$ **.

Table 1. Change in *Chlamydia* PCR load following vaccination : Percentage (and raw number calculations) of koalas that were *C. pecorum* positive at time point 0 (i.e. at initial vaccination time), and then exhibited changes in their *C. percorum* load between either 0 and 6 months, or between 0 and 12 months, post vaccination. Statistically significant effects are shown in bold. Trending ($P < 0.1$) results indicated with *. Grey shading represents groups with more than expected (based on Pearson residuals) for significant results. The changes are categorized as decreasing, stable or increasing (ΔqPCR $\leq$ -100, -99 – 99, and $\geq$ 100 copies/µL respectively).

	Eye (0 vs. 6 months)			Eye (0 vs. 12 months)			UGT (0 vs. 6 months)		
	Decrease	Stable	Increase	Decrease	Stable	Increase	Decrease	Stable	Increase
Control	33% (2)	0% (0)	67% (4)	100% (4)	0% (0)	0% (0)	69% (9)	0% (0)	31% (4)
Vaccinated	50% (5)	40% (4)	10% (1)	71% (5)	29% (2)	0% (0)	88% (7)	12% (1)	0% (0)
X^2	85.677			31.619			45.299		
P	**< 0.001 (0.052 *)**			**< 0.001 (0.496)**			**< 0.001 (0.099*)**		

[a] analysis based on 2 x 2 contingency table Chi-square owing to no individuals with decreasing loads for both control and vaccinated koala

Chapter 6: Antibody and cytokine responses of koalas (*Phascolarctos cinereus*) vaccinated with recombinant chlamydial major outer membrane protein (MOMP) with two different adjuvants

6.1 Statement of contribution of co-authors for thesis by published paper

The authors listed below have certified that:

1. They meet the criteria for authorship in that they have participated in the conception, or execution, or interpretation, of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to (a) granting bodies, and (b) the editor or publisher of Vaccine and
5. They agree to the use of the publication in the student's thesis and its publication on the QUT ePrints database consistent with any limitations set by publisher requirements.

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Contributor	Statement
Shahneaz Ali Khan	Contributed to the project design and over all implementation, conducted the majority of the laboratory work in the project, conducted the analysis and interpretation of the data and wrote the manuscript
Signature	
Date	
Marion Desclozeaux	Contribution to critical reading and editing of the manuscript and Contributed the laboratory work for qPCR and contributed to the overall collaboration
Courtney Waugh	Contribution to critical reading and editing of the manuscript
Jon Hanger	Veterinary expertise and contributed to thorough clinical examination and sampling of koalas in this field study
Jo Loader	Veterinary expertise and contributed to the sampling of koalas and involved in the collaboration and approval of this study
Volker Gerdts	Provided the adjuvant and advised on the components of the adjuvant
Andrew Potter	Provided the adjuvant and advised on the components of the adjuvant
Adam Polkinghorne	Contribution to critical reading and editing of the manuscript
Kenneth Beagley	Contributed to the project design, critical reading and editing of the manuscript
Peter Timms	Designed the initial research plan; involved in experimental planning , design and securing funding; major contributor in collaborating with the industry partners; critical reading, editing and approved the manuscript

Principal Supervisor Confirmation

I have sighted email or other correspondence from all co-authors confirming their certifying authorship.

PETER TIMMS		3/5/2016
Name	Signature	Date

6.2 Abstract

Developing a vaccine against *Chlamydia* is key to combating widespread mortalities and morbidities associated with this infection in koalas (*Phascolarctos cinereus*). In previous studies, we have shown that two or three doses of a Recombinant Major Outer Membrane Protein (rMOMP) antigen-based vaccine, combined with Immune Stimulating Complex (ISC) adjuvant, results in strong cellular and humoral immune responses in koalas. We have also separately evaluated a single dose vaccine, utilising a tri-adjuvant formula that comprises polyphosphazine based poly I: C and host defense peptides, with the same antigen. This formulation also produced strong cellular and humoral immune responses in captive koalas. In this current study, we directly compared the host immune responses of two sub-groups of wild *Chlamydia* negative koalas in one population vaccinated with the rMOMP protein antigen and adjuvanted with either the ISC or tri-adjuvant formula. Overall, both adjuvants produced strong *Chlamydia*-specific cellular (IFN-γ and IL-17A) responses in circulating PBMCs as well as MOMP-specific and functional, *in vitro* neutralising antibodies. While the immune responses were similar, there were fine detailed differences between the adjuvants, particularly in relation to the specificity of the MOMP epitope antibody responses.

Key words: *Chlamydia*, Vaccine, rMOMP, Adjuvants, Koala

6.3 Introduction

Chlamydial infections are responsible for significant mortality and morbidity of mainland koalas (*Phascolarctos cinereus*) and are one major factor threatening the long term future of this iconic species [1-4]. The main species of *Chlamydia* that infects koalas is *C. pecorum*, and virtually all koala populations are infected, with rates ranging from 10% to as high as

90% in some regions [1]. Despite the significant advances in chlamydial research, a prophylactic vaccine to stabilize the population decline caused by chlamydial infections [5] has yet to be fully developed.

Chlamydia is an intracellular bacterium with a unique biphasic developmental cycle, consisting of two developmental forms, the non-dividing, but infectious, elementary bodies (EBs) and the replicative, but non-infectious reticulate bodies (RBs) [6]. It is usually accepted that a host requires the development of a balanced Th1 and Th2 protective immune response to adequately control chlamydial infections [7]. Several small animal studies have confirmed the protective role of IFN-γ secreting CD4+T cells in chlamydial infection [8]. Recently, there is also re-emerging evidence supporting the prominent role of B cells to elicit protective anti-*Chlamydia* antibodies [9]. The primary role of the neutralizing antibodies is to reduce the initial infectious burden and further prevent secondary bacterial infections [10]. Once the bacterium parasitises the host's cells, the cell mediated immune response pathway contributes significantly to protective immunity through IFN-γ secretion [11]. Whilst IL-17A is a strong recruiter of neutrophils which secrete antimicrobial peptides and promote a Th1 immune response against intracellular pathogens [12], other animal studies suggest that IL-17 plays a role in both immune pathology and protection [13].

The chlamydial major outer membrane protein (MOMP) is the leading vaccine candidate in chlamydial vaccine research, and our group has been developing a prototype vaccine utilizing recombinant chlamydial MOMP (rMOMP) as a vaccine antigen for koalas. Although the choice of immunogenic antigen is of prime importance, selecting the right adjuvant to appropriately trigger the immune response is also essential. In this context, we have used two

different adjuvant formulations with differing properties, combined with rMOMP, to vaccinate groups of koalas: ISC (Immune stimulating complex) adjuvant [14-17] or Tri-adjuvant which is a mixture of the three components (Polyphosphazine based poly I: C and host defense peptides) [18].

In our previous koala vaccine trials, the ISC adjuvant was able to induce strong cellular and humoral immune responses [14-17]. However, the ISC adjuvant requires two or three injections to promote a significant immune response. This is logistically problematic for wild koalas, which would need to be tracked and re-captured, or kept in captivity for extended periods of time, increasing the cost of the process as well as the stress experienced by the animal itself. A trivalent adjuvant (Tri-Adj) containing polyphosphazine, poly I: C and host defense peptides, has been developed to be effective with just a single dose [18]. In other species, this adjuvant promoted a Th1 and Th2 balanced immune responses following a single injection [19-23]. In a small preliminary trial in captive koalas (n = 6), we have shown that this adjuvant was safe to use and elicited promising immune responses [18].

In the current study, we evaluated, in detail, both the cellular and humoral immune responses of wild koalas vaccinated with rMOMP, combined either with (a) the single-dose Tri-Adj or (b) three doses of ISC. Firstly, we evaluated the cellular response for each adjuvant by measuring cytokine expression elicited by the peripheral blood mononuclear cells (PBMCs) at defined post-vaccination time points. Secondly, we measured the neutralising antibodies produced by vaccination and mapped the corresponding MOMP epitopes recognized for both cohorts.

6.4 Materials and Methods

Koalas

The koalas used in our study were sourced from a wild population of around 400 animals located in South East Queensland. Prior to vaccination, all animals were examined and those animals that (i) had no clinical evidence of chlamydiosis; and (ii) were negative at conjunctival and genital sites following *Chlamydia pecorum*-species-specific qPCR screening [24] were selected. Two sub-sets of these animals have been vaccinated with an anti-*C.pecorum* vaccine and we analysed a further sub-set of these vaccinated animals in the current study. The first group of 10 koalas (Cindy, Greg, Cherry, Maxwell, Kylie, Paige, Janke, Squeek, Linky and Kelly) (Group A) were vaccinated with chlamydial rMOMP protein (see below for details) mixed with the Tri-Adj. A second group of 5 koalas (Robyn, Pepper, Maya, Hunky Harry and Winnic) (Group B) were vaccinated with rMOMP protein mixed with ISC [17]. At the end of the trial, all koalas were successfully returned to their habitat in accordance with current legislation and the detailed Koala Action Plan for the Moreton Bay Rail Link project. All work was conducted under permission from Queensland University of Technology's Animal Ethics Committee (AEC; Permit # 1200000122), the University of the Sunshine Coast AEC (ANA1380) and Scientific Purposes Permit (WISP11532912).

Vaccines

Both vaccines consisted of *C. pecorum* rMOMP combined with either adjuvant (Tri-Adj or ISC). We combined three rMOMP proteins (A, F and G types) for the vaccine, as described previously [14, 17, 18]. Koala-specific *C. pecorum* MOMP proteins were expressed and purified as per Kollipara et al. [14]. The purified products were used for vaccination and

ELISA assays. After vaccination, the animals were released back into the wild and tracked with a wildlife telemetry system (K-Tracker, LX Solutions Pty Ltd). The ISC vaccinated koalas were re-captured at 1 monthly interval to receive the 2nd and 3rd dose of the vaccine and a veterinary health examination.

Samples

Aluminium shafted cotton-tipped swabs (Copan, Interpath Services, Melbourne) were used to collect samples from the conjunctiva of the left and right eye, as well as the urogenital sinus (prostatic urethra in males), as previously described [18]. These swabs were used for measuring the *C. pecorum* infection load using a *C. pecorum*-species-specific qPCR targeting the 16S rRNA gene [24]. Blood samples were obtained from the cephalic vein into EDTA-containing tubes and stored at 4° C for processing within 24 h of collection, to obtain PBMCs. After centrifugation at 1000 rpm for 5 mins, plasma was separated and used for ELISAs and *C. pecorum in vitro* neutralisation assays. The samples were collected at 0 (pre-vaccinated), 2 and 6 months post vaccination.

Cytokine assays

The blood samples were centrifuged within 4-8 h of collection to separate the plasma. The PBMC were isolated by centrifugation on Ficoll-paque gradients (GE Healthcare, Rydalmere, Australia) washed and suspended in 1ml RPMI 1640 T cell media supplemented with 5% foetal calf serum, antibiotics and β-mercaptoethanol (0.001M) (Sigma) at a concentration of $2x10^6$ cells/ml. A 500 µl aliquot of cell suspension was used as the pre-stimulation sample. The remaining cells were then stimulated with either mitogens (Ionomycin and PMA combination)[25] or UV-inactivated *C. pecorum* EBs. After stimulation and incubation at 37°

C with 5% CO_2, the cells were collected at 12 and 24 h post-stimulation time points. RNA extraction and cDNA synthesis were completed for all these pre- and post-stimulation samples according to our previously published protocol (21). The end products were utilized in qPCR assays to determine the mRNA expression level as fold change for interferon gamma (IFN-γ), interleukin 17A (IL-17A), interleukin 10 (IL-10), tumour necrosis factor alpha (TNF-α) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [25-27]. GAPDH was used as reference to normalise IFN-γ, IL-17A, IL-10 and TNF-α using the $2^{-\Delta\Delta CT}$ method (ΔΔCT = (Ct of target gene - Ct of GAPDH) at 12 or 24 h time point – (Ct of target gene – Ct of GAPDH) at 0 time point [28].

C. pecorum specific ELISA

Enzyme linked immunosorbent assays were performed using purified rMOMP as per Kollipara et al. [14] and Khan et al.[18] on the plasma samples collected at 0, 2 and 6 month time points post-vaccination.

C. pecorum MOMP peptide ELISA

We initially screened the plasma to identify the reacting epitopes for individual animals, using the methods described previously [9]. Then we measured the individual peptide concentrations as determined using our previously described ELISA methods [18]. Instead of using the whole rMOMP protein, we used selected peptides for coating the ELISA plates at a concentration of 2µg/well in PBST. Post-incubation, the wells were washed 3x with PBST and the plasma sample was serially diluted two fold at 1:200 dilution initially, and incubated at 4° C overnight. Plates were then washed 3x in PBST and a sheep anti-koala IgG (1:8000 in PBST;[16]) was added. At this point, plates were incubated for a further one hour at room

temperature. After a further three washes (PBS-T), HRP-conjugated rabbit anti-sheep IgG (1:1000, Southern Biotech / Millipore, North Ryde, Australia) was added to wells and incubated at room temperature for 1 hr. Post incubation, plates were washed 4x with PBS and 50 µl ABTS [2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), Southern Biotech, Alabama, USA] solution was added and incubated for 10 mins to observe the greenish color development. The reaction was stopped with 1M sulphuric acid following color observation. The optical density was measured at 405 nm wavelength and the data was transformed into excel sheet for later analysis.

Koala-specific *C. pecorum* neutralising antibodies

We conducted *in vitro* neutralisation assays using the methodology of Kollipara et al. [14] either on whole plasma or on plasma collected at 0, 2 and 6 month time points which had been pre-adsorbed with one or more individual peptides [14]. All plasma samples were diluted at 1:10 prior to assay. The background neutralisation was determined by using koala plasma that was *Chlamydia* negative. Percentage neutralization was then determined by subtracting this background from each individual to determine the final neutralisation. The results were expressed as fold change neutralisation.

C. pecorum MOMP peptide mapping

Biotinylated Pepscan ELISA was performed as previously described [9] to identify the specific rMOMP epitopes produced by each vaccine in animals receiving either the ISC or Tri-Adj adjuvants. Briefly, we designed 88 peptides with 15-mer peptides that spanned the full length of koala *C. pecorum* MOMP F protein and used these individually in ELISA assays (or grouped) as described above. The background for each plasma sample was

calculated from the mean plus twice the standard deviation of the negative wells (no plasma added). We scored samples with an absorbance value greater than 0.5 as a positive response. In subsequent experiments, we utilised only the positive peptides to coat the streptavidin plate at a concentration of 2μg/well and performed the standard ELISA as described.

C. pecorum MOMP-peptide specific neutralising antibodies

We performed three types of neutralising assays by using the (a) whole plasma at 1/10 dilution for either Tri-Adj or ISC cohort, (b) whole plasma at post-adsorption against either peptide 58 and 77 for tri-adjuvant or at post-adsorption against epitope 4 for ISC cohort and finally (c) whole plasma at post-adsorption against either epitopes 4, 28, 41, 42, 58, 59 and 77 for Tri-Adj cohort or 4, 28, 41, 42 for ISC cohort. We utilised the previously described novel protocol for peptide adsorption [9].

Statistical analysis

Statistical analyses were performed using Graph-Pad Prism version 6 (Graph Pad Software, La Jolla, CA, USA) and the P value for significance was set at ≤ 0.05.

6.5 Results

There was a non-significant trend towards stronger IFN-γ and IL-17A responses in animals immunised with the Tri-Adj compared to ISC immunised animals

To evaluate differences in the immune response of koalas vaccinated with a *C. pecorum* rMOMP-vaccine adjuvanted with either Tri-Adj or ISC, we vaccinated a cohort of koalas that were clinically healthy at the time of vaccination and were *Chlamydia* PCR negative at both

urogenital and ocular sites (data not shown). Immune profiling of these vaccinated animals revealed that 60% of the animals in both groups produced IFN-γ at 2 or 6 months post vaccination in response to stimulation of PBMCs with UV-inactivated EBs (elementary bodies) (6 out of 10 for Tri-Adj and 3 out of 5 for ISC adjuvant). For those animals who's PBMCS expressed IFN- γ in response to stimulation, the level of IFN-γ expression varied from 2.73 to 17.89 for Tri-Adj and from 2.08 to 12.67 for ISC (Fig. 1). We also observed differences among the responders between the 2 month and 6 month time points. For the Tri-Adj responders the highest expression was observed at 2 months, whereas, for ISC responders the highest IFN-γ responses were at the 6-month time point. Overall, the IL-17A responses were lower than IFN-γ, and only 40% of animals (4/10 Tri-Adj; 2/5 ISC) produced IL-17A responses to stimulation above 1.0 fold. We did not observe any measurable expression for the anti-inflammatory cytokine, IL-10 and TNF-α following stimulation of collected PBMCS from animals in either cohort (Fig. 2 A-D).

The kinetics of the total antibody (IgG) titres was similar in both cohorts, though there was an increased trend towards higher plasma IgG titres in ISC cohorts

Both vaccine formulations elicited strong anti-MOMP antibody levels following vaccination. The Tri-Adj cohort produced titres of around $5x10^5$ at 2 months post-vaccination, which persisted up to 6 months. The ISC cohort produced a similar average titre at 2 months ($7x10^5$) which increased (to $9x10^5$) by the 6 months time point (*p* value 0.302) (Fig. 3A). We also measured the antibody responses to individual peptides (selected ones) by ELISA (Fig 5). The titres for the individual epitopes varied from $0.3x10^3$ to $2.8x10^3$ EPT. Interestingly, there

was very little difference in titres for the individual epitopes, except for epitope 77 in a single koala (Kelly) (Fig. 5).

Similar *C. pecorum* specific neutralising antibody potential was produced by both adjuvants

To compare the function of antibody responses induced by either vaccine formulation, *in vitro* neutralisation assays were performed with plasma from the Tri-Adj and ISC cohorts at 2 and 6 months post-vaccination. All samples were diluted 1:10 prior to testing their neutralising ability on *C. pecorum* infected cell culture mono-layers. Both adjuvant cohorts produced almost identical *in vitro* neutralisation levels, with both groups having increased neutralisation levels at 6 months compared to 2 months post vaccination (Fig. 3B).

Epitope mapping identified two distinct anti- *C. pecorum* MOMP peptide antibody profiles for the two adjuvant groups.

We used the Pepscan approach [9] to examine the epitope specificity of the plasma antibody response to vaccination in our Tri-Adj versus ISC adjuvant groups. In total, four C. pecorum MOMP peptides (4, 28, 41, and 42) were recognized in our *C. pecorum* peptide ELISA from animals in each cohort with an additional three peptides recognised by koalas receiving the Tri-Adj formulation only (58, 59 and 77). There was variability in the responses to these individual peptides with peptide 4 recognised by 80% (4 out of 5) of the ISC cohort but only by 10% (1 out of 10) of the tri-adjuvant cohort. For the other epitopes, none were recognised by 100% of the animals in any cohort, although the most-broad recognition by the animals

was with epitopes 77 (8/10 of tri-adjuvant animals), 58/59 (5/10 tri-adjuvant animals), 41/42 (5/10 tri-adjuvant cohort) and 28 (4/5 ISC animals) (Fig. 4).

The vaccine induced anti-epitope antibodies had neutralising ability, either individually or in synergy with other epitopes

We examined the contribution of antibodies against individual epitopes or groups of epitopes, to the observed *in vitro* neutralisation effect. We compared (a) whole plasma versus (b) plasma pre-adsorbed against the most recognized peptides 58, 77 for Tri-Adj, and peptide 4 for ISC versus (c) plasma pre-absorbed against epitopes 4, 28, 41, 42, 58, 59, 77 for Tri-Adj and 4, 28, 41, 42 for ISC. We evaluated the neutralising ability of each of these pre- and post-absorption samples and compared the relative reduction of neutralisation ability in each case (Fig. 6 B, E). We found that most (if not all) of the individual anti-epitope antibodies contributed to *in vitro* neutralisation. In the case of the Tri-Adj vaccinated animals, anti-58 and anti-77 epitope antibodies made a major contribution to the *in vitro* neutralisation effect (white bars in Fig 6B). The effect of these antibodies was confirmed with animal “Cherry” (did not produce any anti-58 antibodies) and animal “Squeek” (did not produce any anti-77 antibodies) as the *in vitro* neutralisation level for these animals was not reduced following absorption against 58 or 77 peptides. We also observed significant *in vitro* neutralisation by antibodies against peptides 4, 41/42 and 28, with anti-peptide 4 antibodies (especially in the ISC cohort), having a major effect (Fig 6E).

6.6 Discussion

Our previous work suggested that the koala's immune system is able to mount both effective cellular and humoral immune responses against a rMOMP vaccine, when administered in combination with two different adjuvant systems [14-18]. While both adjuvant vaccines look promising, one requires two or three doses (ISC) while the other is a single administration vaccine (Tri-Adj). We therefore decided to directly compare the immune responses of the two vaccine formulations using the same rMOMP antigens to vaccinate koalas from the same wild population.

Studies with the mouse model of *C.muridarum* show that an IFN-γ response is required for adequate protection against chlamydial infections. While there is no direct evidence yet for protection against *C. pecorum* infections in koalas, vaccine development should aim for a strong IFN-γ response. We found that both vaccine formulations induced good IFN-γ responses in 60% of animals that lasted for up to 6 months. No significant difference could be seen in the specific IFN-γ response induced by the single dose Tri-Adj formulation or the ISC formulation. IFN-γ activity is the hallmark of the Th1 immune response against chlamydial infection and IFN-γ gene knockout mice are indeed unable to resolve the infection [29]. Despite the promising IFN-γ response in some animals (60%), not all koalas produced a detectable IFN-γ response. The animals in this trial are outbred animals and this highlights key Major Histocompatibility Complex (MHC) considerations for future vaccine development. Genetic differentiation and structure analysis has revealed that the koala's MHC-II gene is more diverse in koalas in the northern states of Queensland and New South Wales, compared to the southern states of Victoria [30, 31]. The higher MHC-II diversity could be the potential cause of the variable immune response within this group of koalas.

In addition to IFN-γ, IL-17A has been suggested as an important cytokine for chlamydial infection, both for protection, but potentially also in disease pathology [13, 32]. We observed *Chlamydia*-specific IL-17A responses for 40% of koalas with both vaccine formulations. Whilst, recent studies in koalas [27] and women [33] reported that strong expression of IL-17A has been associated with clinical chlamydiosis and chlamydial cervicitis, the IL-17-/- mice had shown less pathological lesions compared to BALB/c [13]. Moreover, an elevated IL-17A response has been observed in clinical chlamydial infection in mouse model [34]. Though the mechanism of IL-17A in pathogenesis is unclear, this study confirms both vaccines can induce expression of this cytokine.

While we did not observe any measurable anti-inflammatory cytokines response in either group, still their role in chlamydial immunity and pathogenesis is controversial. In general, IL-10 suppresses the secretion of various pro-inflammatory cytokines involved in chlamydial pathogenesis [35]. Furthermore, in the mouse model, the IL-10 dominated response has been attributed with susceptibility to chronic infection [36]. A similar observation has been seen in trachoma infected populations [37]. The higher expression of the IL-10 gene promoter has been associated with increased chlamydial infection and disease severity [38]. Similarly, a higher level of IL-10 has been linked to *C. trachomatis* infertility [39, 40] and tubal damage in women [41]. However, koalas with clinical chlamydiosis, expressed IL-10 in variable levels, with some animals showing higher level of expression similar to IFN-γ [25, 27]. In a similar fashion, the role played by TNF-α in chlamydial infection has provided disparate results. However, TNF-α has been linked to an initial clearance of primary infection but challenge infection elicited immune-pathology in the mouse [42] and guinea pig model [43].

In the mouse model studies showed TNF-α produced by CD8+ T cells, promote inflammation in the oviduct following *C. muridarum* infection [44] but CD4+ T cells producing IFN-γ and TNFα are generally immune-protective. In contrast, reduced chlamydial shedding following challenge infection in vaccinated mice, has been attributed to the co-expression of TNF-α and IFN-γ [45].

While cytokines are considered to be the major immune mechanism for protection against chlamydial infections, antibodies continue to be considered just as important. In fact, recent data confirmed the protective roles of antibodies in chlamydial infection in koalas and other animal studies [46, 47]. If antibodies do play a role in protection, then it will be via their neutralisation role. We therefore measured the *in vitro* neutralisation ability of plasma antibodies from animals immunised with the two adjuvants. We evaluated both their total neutralisation ability but we also determined which peptides within the MOMP protein the antibodies were directed against and which of these were the most important for the neutralisation effect. This produced very interesting and promising results for the neutralisation ability of plasma from vaccinated koalas.

Firstly, both adjuvants produced antibodies that were equally neutralising. This confirms that MOMP has B cell epitopes that can be neutralising, validating it as a good vaccine target. Interestingly, the adjuvants resulted in a different, but overlapping, set of vaccine-induced epitopes. Three peptides were recognised by both adjuvants (4, 28, and 41/42), but two additional epitopes (58/59 and 77) were solely recognised by Tri-Adj-immunised animals. The adsorption experiments nicely confirmed that several anti-epitope antibodies contributed to the *in vitro* neutralisation effect. Studies in the non-human primate model utilising native

MOMP formulations had previously shown serovar- specific immune response either to homologous serovars [48] or cross-reacting to the closely related heterologous serovars [49]. Interestingly, in this study, the vaccine-induced epitopes recognised are all located in the conserved domains suggesting their role in cross-reactive recognition against diversified MOMP genotypes. Several vaccine studies have used the native form of MOMP, arguing that MOMP in its native should elicit a more robust immune response [48]. However, this study suggests that rMOMP is capable of generating neutralising epitopes in koalas. Nonetheless, 80% of the animals responded to epitope 77 in the variable region, but did not result in extra neutralising capacity.

In summary, both the adjuvants induce Th1-biased immune responses with neutralising antibodies. It is promising that the single dose Tri-Adj is able to produce a comparable immune response to the two or three-shot ISC up to 6 months time point. Tri-Adj has proven to be an effective adjuvant system for koala-*Chlamydia* vaccine design, and a practicable solution to eliminate multiple vaccination events. All of the surviving animals in our original study [16] that were immunised with the ISC adjuvant have high plasma antibody levels and memory CD4+ T cells 8 years after vaccination, while we don't yet have similar data for the Tri-Adj. The identification of key epitopes (for the development of neutralising antibodies) enables future studies to focus on including these, or to develop specific assays to evaluate vaccine effectiveness.

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Conflict of interest

The authors declare that they have no conflict of interest.

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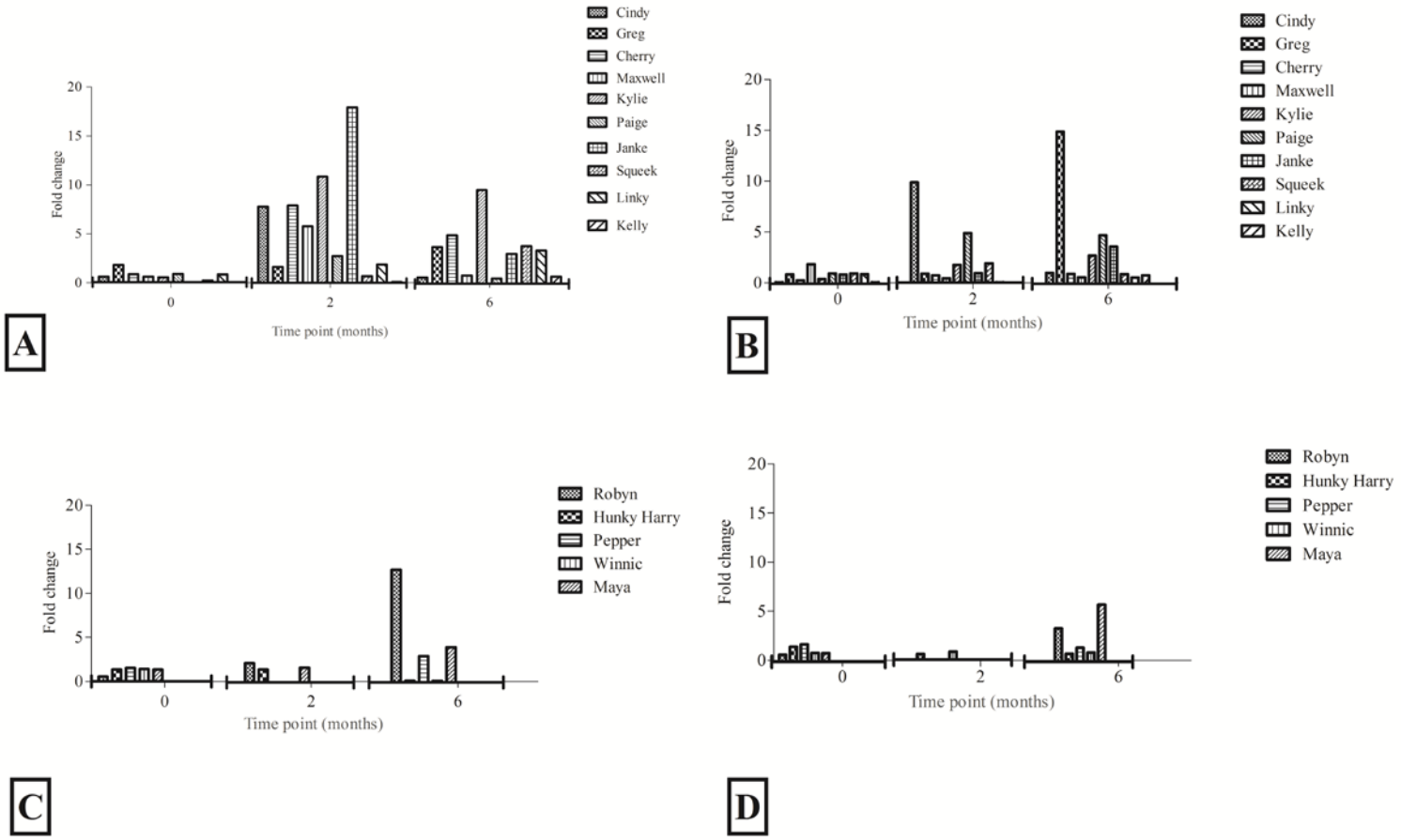
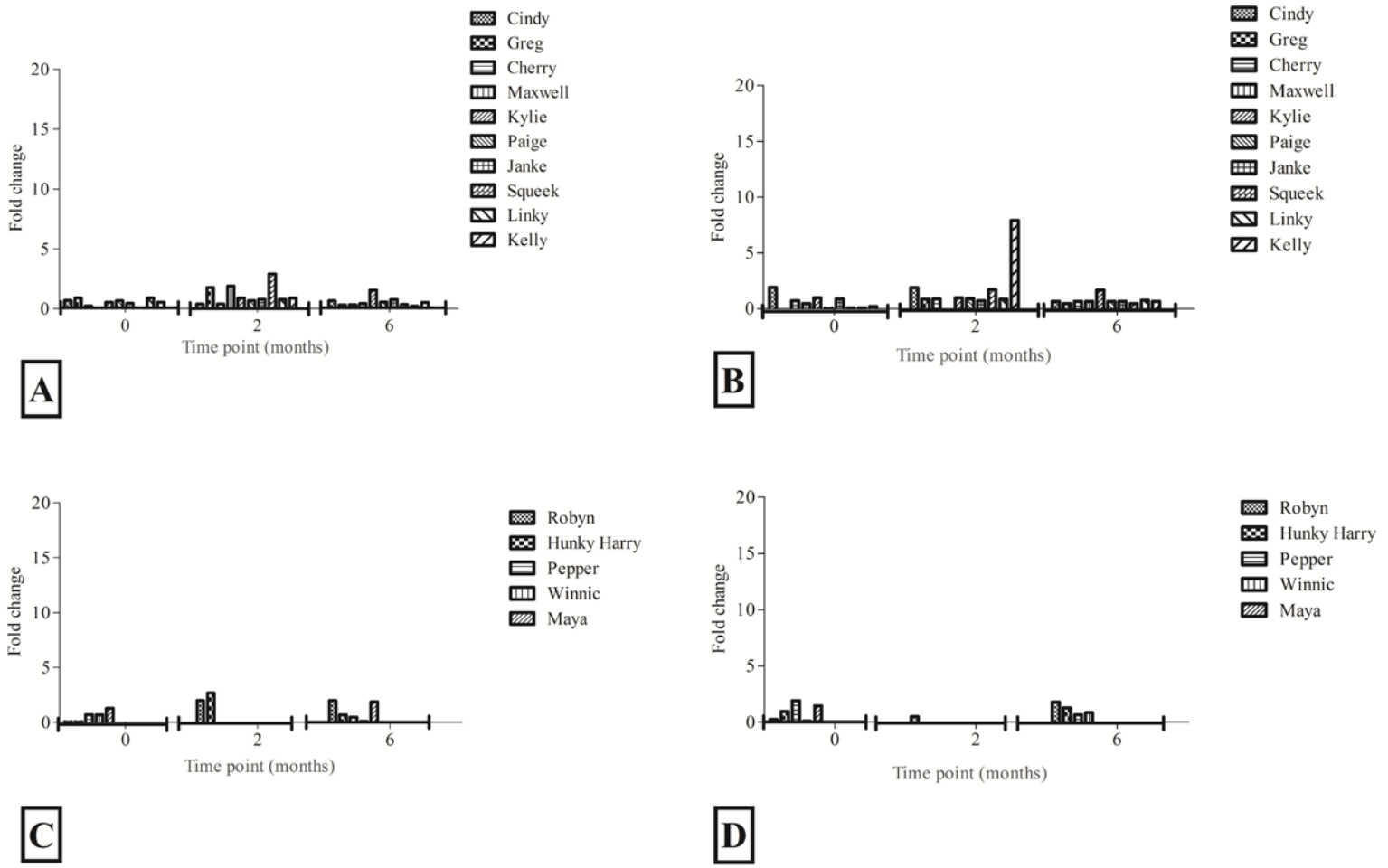


Antibody and cytokine responses of koalas (*Phascolarctos cinereus*) vaccinated with recombinant chlamydial major outer membrane protein (MOMP) with two different adjuvants

Fig. 1 IFN-γ (A, C) and IL17A (B, D) gene expression in koala PBMCs stimulated with UV inactivated *C. pecorum* at 0, 2 and 6 months post vaccination. The Tri-Adj (A, B) and ISC (C, D) cohort's response are presented together (Fig. 1 A-D). Results are expressed as fold increase compared to internal control gene GAPDH



Antibody and cytokine responses of koalas (*Phascolarctos cinereus*) vaccinated with recombinant chlamydial major outer membrane protein (MOMP) with two different adjuvants

Fig. 2 IL-10 (A, C) and TNFα (B, D) gene expression in koala PBMCs stimulated with UV inactivated *C. pecorum* at 0, 2 and 6 months post vaccination. The Tri-Adj (A, B) and ISC (C, D) cohorts are presented together (Fig. 2 A-D). Results are expressed as fold increase compared to internal control gene GAPDH

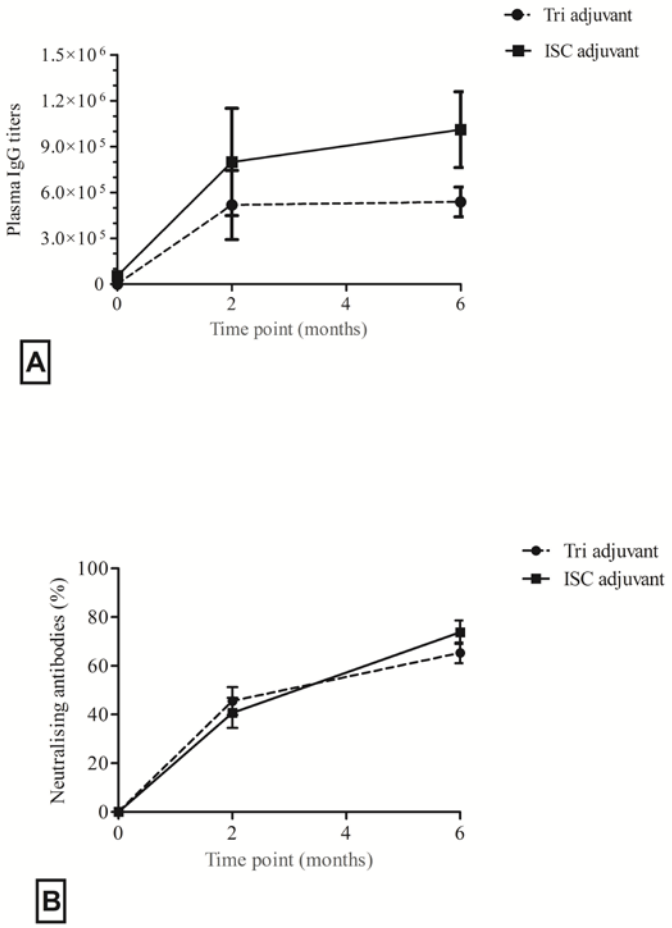


Fig. 3 rMOMP specific IgG in plasma of vaccinated koalas was assayed by ELISA at 0, 2 and 6 months post vaccination. IgG levels are expressed as end-point titers (EPT) and represent the mean ± SD of 10 and 5 koalas in the Tri-Adj and ISC cohort respectively (Fig. 3A). Vaccine induced *C. pecorum* percent neutralisation in plasma is presented (Fig. 3B) compared to pre-immunisation samples. All samples were assayed at 1:10 dilution and *C. pecorum* EBs (50,000 IFU) were added to samples. The results are expressed as the percentage neutralisation of post-immunized samples compared to that of the pre-immunized and non-infected samples. Results are expressed as the mean ± SD of 10 and 5 koalas in the

tri adjuvant and ISC cohort respectively. There was no significant difference between the two cohorts.

Tri adjuvant cohort	CD	VD F1	CD	VD F2	CD	VD F3	CD	VD F4	CD	VD A	VD G	VD H	CD
Cindy									58, 59		77		
Greg			28								77		
Cherry	4		28										
Maxwell			28				41				77		
Kylie							41		59		77		
Paige							41		58, 59		77		
Janke									58, 59		77		
Squeek							41, 42						
Linky							41				77		
Kelly									58		77		
ISC cohort	CD	VD F1	CD	VD F2	CD	VD F3	CD	VD F4	CD	VD A	VD G	VD H	CD
Robyn	4						41, 42						
Hunky Harry	4		28										86
Pepper	4		28										
Winnic	4		28										
Maya			28				41						

Fig. 4 Epitope mapping of antibodies in plasma samples at 6 months post vaccination as determined by Pepscan assay. CD: MOMP Conserved domain; VD F1: Variable domain 1 for MOMP F; VD F2: Variable domain 2 for MOMP F; VD F3: Variable domain 3 for MOMP F; VD F4: Variable domain 4 for MOMP F; VD A: Variable domain 1, 2, 3, 4 for MOMP A; VD G: Variable domain 1, 2, 3, 4 for MOMP G; VD H: Variable domain 1, 2, 3, 4 for MOMP H.

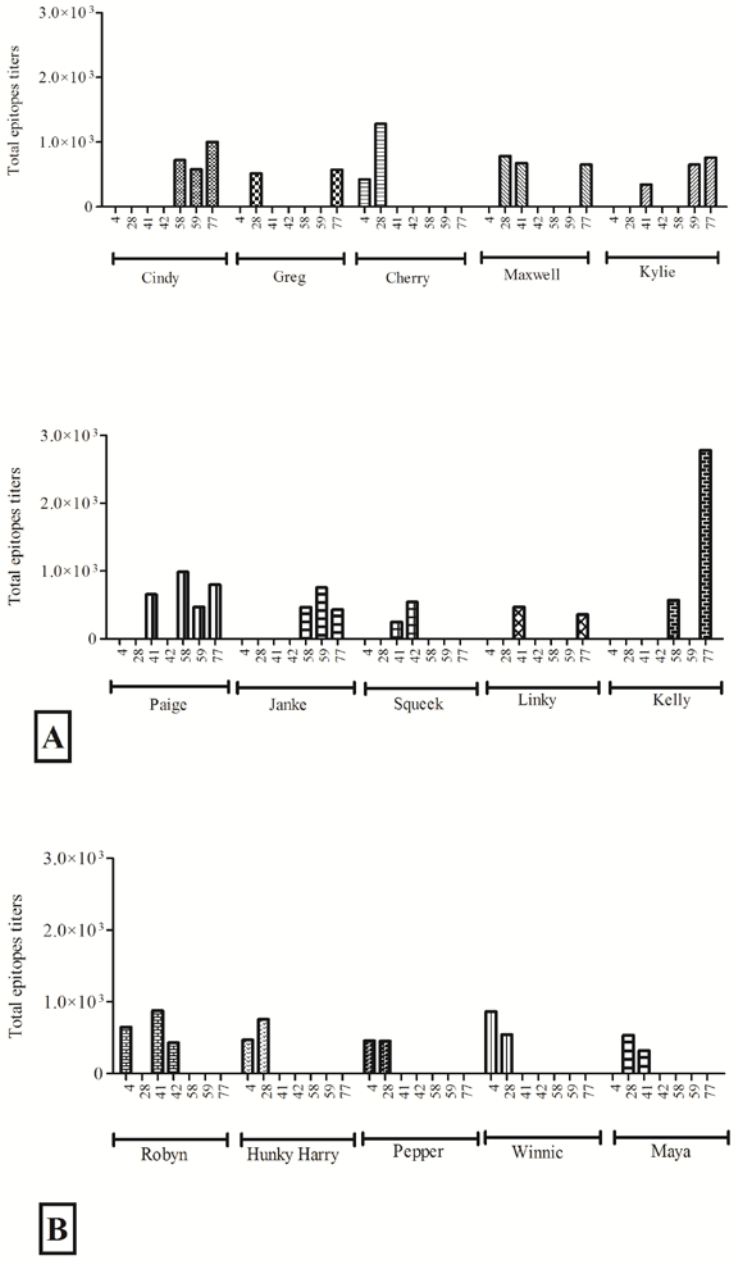
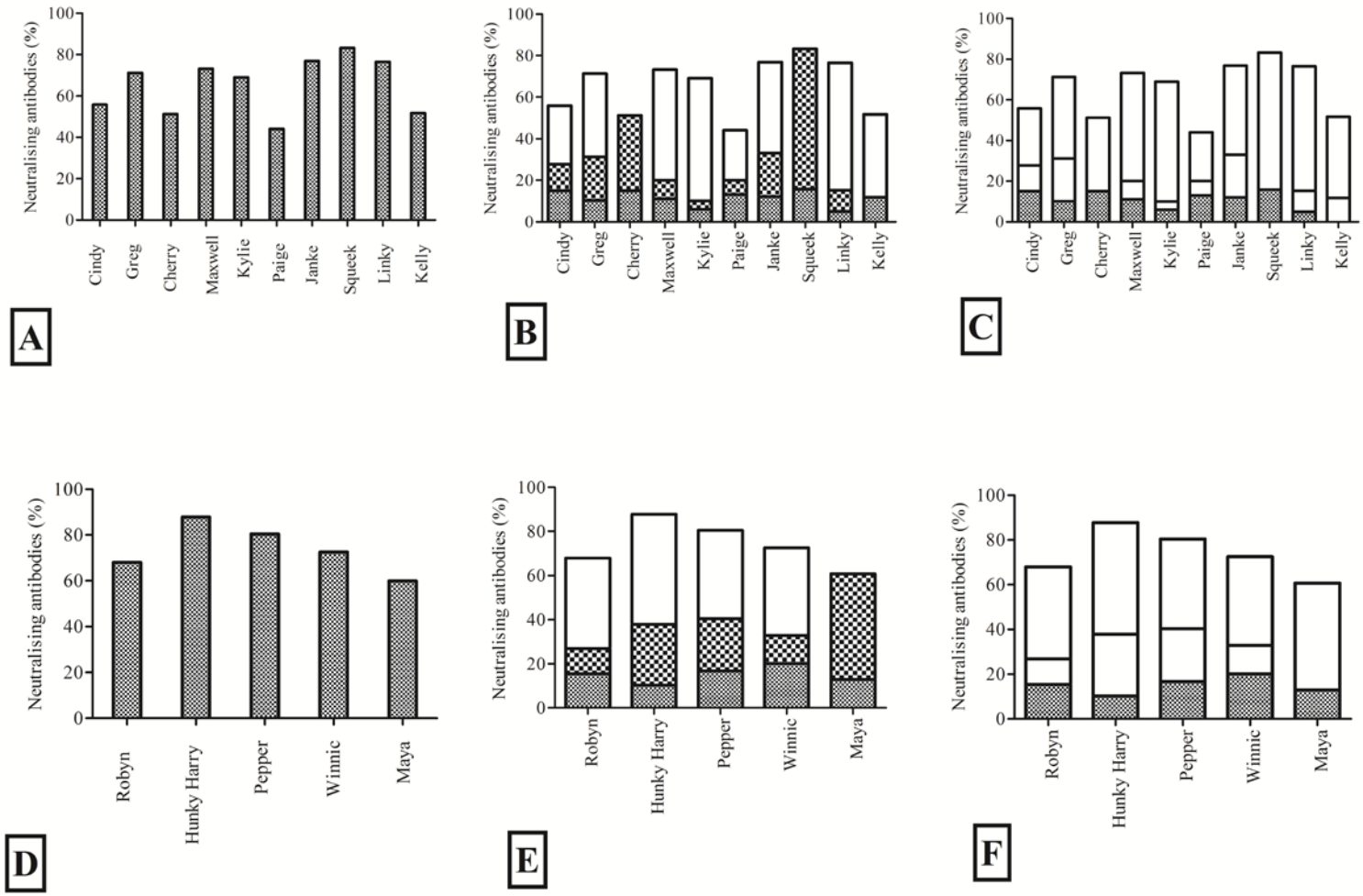


Fig. 5 ELISA titers against individual peptide. Panel A (10 animals) for the tri-adjuvanted vaccinated animals and panel B (5 animals) for the ISC cohorts. We did not consider epitope 86 as this epitope was detected in naturally infected koalas in our previous study.



Antibody and cytokine responses of koalas (*Phascolarctos cinereus*) vaccinated with recombinant chlamydial major outer membrane protein (MOMP) with two different adjuvants

Fig. 6 *In vitro* neutralisation levels of plasma from vaccinated koalas (A) whole plasma at 1/10 dilution for Tri-Adj cohort, (B) whole plasma after absorption against peptides 58 and 77, or (C) whole plasma after adsorption against peptides, 4, 28, 41,58, 59, and 77. Separately, the *in vitro* neutralisation of plasma from ISC cohort (D) whole plasma at 1/10 dilution, (E) whole plasma after adsorption against peptide, 4, (F) whole plasma after adsorption against peptides, 4, 28, 41 and 42. The neutralising antibodies were presented against individual animals and all samples were diluted at 1:10 dilution and *C. pecorum* EBs (50,000 IFU) were added to samples. The results are expressed as the percentage in neutralisation of immune samples compared to that of the pre-immune and non-infected samples. The reductions of neutralising antibodies are presented as empty spaces in the bar.

Chapter 7: General discussion

General discussion

The koala (*Phascolarctos cinereus*) is an iconic Australian tree-dwelling marsupial and the only surviving member of the Family *Phascolarctidae*. This animal has a unique position in the rich biodiversity of Australia as well as its valued contribution to the tourism industry. However, in the early 1800s, during the European settlement in Australia, its survival was threatened by a number anthropogenic risk factors [1]. The Australian Federal Government has recently recognised koalas as a threatened species across two-thirds of their range, in New South Wales (NSW) and Queensland (QLD) populations which have shown the largest decline [2]. A recent study showed that QLD, NSW and the Australian Capital Territory (ACT) koala populations have been declining in numbers, with high mortality rates being attributed to domestic dog attacks [3], vehicle strikes [4], pronounced urbanisation [2] and chlamydial disease [5]. *Chlamydia* is continuing to drive the overall koala population decline through increased mortality and reduced fecundity [2]. Disease modelling suggests that halting chlamydial infection would be a significant management strategy that could potentially help reverse the population's declining trends [6].

Chlamydiosis is unfortunately a very common reason for the admission of wild koalas to koala hospitals and koala care centres, being the second most frequent reason for such admissions [7]. Koala chlamydial infection is widespread in most wild koala populations, and has been associated with ocular and reproductive tract diseases, including kerato-conjunctivities, rhinitis, inflammation and fibrosis of the urinary bladder and the upper genital tract. There are different grades of infection at both anatomical sites [8]. The chronic active form of chlamydiosis results in the shedding of the highest number of infectious particles, posing serious threat to future transmission [9]. However, the asymptomatic nature

of infection, as also seen in humans, makes it difficult to treat with systemic antimicrobial drugs [10]. Therefore, a vaccine would be an ideal option to effectively control this infection and potentially increase the lifespan of koalas [11].

In the process of vaccine development, the QUT research group identified several key priorities, including; (a) the safety and immunogenicity of rMOMP protein in koala-*Chlamydia* vaccine design [12], (b) matching of the natural diversity seen in *C. pecorum* strains among naturally infected koalas, and how this might relate to vaccine development [13], (c) the suitability of a recombinant MOMP vaccine for use in healthy as well as in naturally infected and diseased koalas [14], (d) the cross-reactive recognition of the single MOMP based vaccine against multiple strains of *C. pecorum* infection [15] and finally, (e) MOMP epitope specificity of the antibody response following infection and vaccination [16]. While vaccine research in koalas is promising, effort is still needed to address issues such as; (a) developing a simpler-to-administer, preferably single dose, vaccination strategy, (b) understanding the detailed mechanisms that underpin both humoral (epitopes mapping and neutralisation) and cellular immunity (cytokines response specifically IFN-γ) in both naturally infected and more importantly, vaccinated koalas, including the role of the adjuvant, (c) evaluating the protective effect of a prototype rMOMP vaccine against the course of infection in wild koalas. This thesis has addressed all of these key research areas.

Initially, this thesis investigated the feasibility of a single dose tri-adjuvant to elicit strong and long lasting cellular and humoral immune responses (Chapter Three). The rMOMP of *C. pecorum* is a promising vaccine candidate that has been evaluated in several koala vaccine trials [12, 14-17]. The immunogenic nature of MOMP has been studied in detail as both

recombinant and native forms, in different animal and human studies [18-24]. Chlamydial MOMP, with it's highly disulphide cross-linked structure, comprises 60% of the outer membrane surface of chlamydial elementary bodies (EBs). However, MOMP has been suggested to have multiple key roles in chlamydial infection and transmission through protection against the host environment, defense against the host's immune response and attachment to the host's target cells. It contains multiple B cell and T cell epitopes, inducing both neutralising antibodies as well as a strong T cell response [25, 26]. Even though MOMP is an immune-dominant component in vaccine design, it has been reported that native MOMP is more suitable for vaccine development [18, 24]. Some of the potential limitations of using rMOMP protein for vaccine development, include, (a) inability to elicit cross-serovar protection and (b) the requirement of adequate tertiary structure to restore the required level of immunogenicity [25].

While MOMP is the leading subunit vaccine antigen and has shown good promise, the adjuvant component is equally important and further effort is still needed. Adjuvants are an important component of vaccine formulations and have major functions for enhancement of antigen uptake and presentation to the secondary lymphoid tissues. These immunomodulatory substances have multiple activities, including depot effects, antigen delivery, recruitment of the specific immune cells to the site of immunisation and maturation of antigen presenting cells [27].To increase the effectiveness of koala chlamydial rMOMP, several adjuvants have previously been evaluated, including, Alhydrogel, Titermax Gold, and Immune Stimulating Complex (ISC) in the design of a chlamydial vaccine for koalas [12]. Several immunisation studies have used ISC adjuvant and these have provided the best immune protection obtained to date. One disadvantage of ISC is that it requires multiple immunisations [14,15, 17]. This

requirement can cause unnecessary stress to the koalas through repeated capture and handling. The koalas also must be held for a longer period of time in captivity, wildlife hospital or wildlife care centre which would be logistically and financially challenging. Therefore, to overcome the limitations of multiple vaccination schedules, we evaluated a tri-component adjuvant containing polyphosphazine, poly I: C and host defence peptide, to generate long lasting cellular and humoral immunity in koalas following a single vaccination

This is the first study in koalas that utilised these novel adjuvant components. Therefore, to evaluate the safety of this tri-adjuvant vaccine formulation, we monitored the koala's health following the administration of the vaccine and throughout the study period. There was no evidence of side effects at the injection site and also no evidence of adverse pathology during the 54 weeks trial period. For evaluating the specific chlamydial cellular immune response, we measured the proliferative response of the PBMC's (peripheral blood mononuclear cells) stained with CFSE (carboxyfluoresceion succinimidyl ester) expressed as the percentage proliferation rate (%). The PBMC's responses were measured against rMOMP and UV-inactivated chlamydial *C. pecorum* EBs. There were no observed differences for proliferation percentage between single and double dose vaccine regimes utilising the tri-adjuvant combined with rMOMP. We also evaluated the humoral immune responses by measuring the IgG antibody titers in plasma and mucosal secretions and again were no significant differences for IgG titers EPT value between two cohorts of animals, irrespective of the anatomical sites (urogenital and conjunctival secretions) analysed. We also measured the *C.pecorum* EB-specific neutralising antibodies in the systemic circulation, ocular and vaginal mucosal secretions. While there were no statistically significant differences between single dose and two dose vaccine cohorts, it was interesting and a promising aspect of this particular

study, that the tri-adjuvant was able to stimulate the neutralising IgG secretions at both the ocular and urogenital tract sites.

The tri-adjuvant that we used in our koala vaccine trial contained 3 immune-stimulating components. The adjuvant mix or individual components have previously been evaluated in different animal models [28, 29]. Synthetic analogue double stranded RNA components such as poly I: C, have been previously tested as an effective immune inducer in several animal species [28, 29]. Poly I: C promotes an immune response via two distinct pathways involving, TLR3 activation and RIG-I/MDA-5, triggering the production of IL-12, type I IFNs production, improved MHC class II and antigen presentation enhancing T and B cell immunity [30]. The presence of type I interferons can enhance antibody titers, increase IFN-γ secretion levels and elicit the generation of long lived memory immune responses [31]. A recent study in humans suggested that IFN-γ can directly induce Th1 cell differentiation, which is critically important for developing cellular immunity against intracellular pathogens [32]. Indeed, IFN-γ contributes to the induction of cytotoxic T cells, activating the antigen presenting cells and thereby promoting the development of Th1 type cellular and humoral immune responses [33, 34].

The tri-adjuvant component PCEP (Polyphosphazine) is a biodegradable polymer and has been shown to enhance mucosal antibody responses (IgG and IgA) in mouse models to a number vaccines when administered parenterally, including influenza, hepatitis B surface antigen, herpes simplex virus type 2 glycoprotein D and cholera toxin [35-37]. PCEP, in combination with influenza antigen, elicits a strong mucosal IgA response when administrated through either the respiratory, nasal or vaginal routes [38]. In addition, PCEP

was shown to induce a mixed Th/Th2 immune response with long lasting and high titers of antibodies in a mouse model [39]. Studies in mice showed the role of PCEP to enhance Th1 type humoral and cellular immune responses for bovine respiratory virus [40]. These mice developed mucosal immune responses, as was the evident from increased production of vaccine antigen specific IgG and IgA in lung tissue culture. Indeed, the increases in systemic and mucosal IgG, and in particular the neutralising IgA, were the most critical aspect of this adjuvant effect. The reason behind this immune response is the production of IL-12 and IFN-γ cytokine responses [41, 42]. Additionally, this Th-1 biased immune response elicited IgG2 antibodies. In a pig model, this PCEP induced IFN-γ based cellular immunity associated with an overall balanced immune response [43]. Similar enhancement of a balanced immune response has been reported with co-administration of PCEP and CpG ODN in a bovine herpes virus vaccine in ruminants (cattle and sheep) [44].

The peptide components of this tri-adjuvant are positively charged with hydrophobic residues that exhibit immune modulatory properties in different animal studies [27, 45]. The adjuvant activities of these HDP (host defense peptides) include PBMC recruitment and activation, increased production of pro-inflammatory cytokines and co-stimulatory molecules on antigen presenting cells which promote phagocytic activities [46]. Previous studies demonstrated that CpG and HDP complexes have potent adjuvant activities in stimulating B cell proliferation and antigen specific antibody production [47, 48]. A recombinant chlamydial protease like activity factor (CPAF) vaccine formulated with CpG and HDP stimulated strong humoral and cellular immune responses with the characteristics of a Th1-biased immune profile [45]. Detoxified pertussis toxin (PTd) in combination with HDP-CpG leads to a 100-fold increase in total IgG levels. Indeed, PTd co-administered with PCEP, HDP and CpG resulted in

increased levels of both IgG2a and IgG1 antibodies in mice and pigs, after just a single dose of vaccination [49]. These studies suggest that HDP in combination with PCEP and TLR3 ligand agonist complex, bridges innate and adaptive immune response to provide a balanced Th1 and Th2 response [46]. The immune responses that we observed in koalas to rMOMP combined with the tri-adjuvant were similar to the previously described study and look very promising.

While a koala-specific IgA reagent to measure this secretory antibody at mucosal surfaces was not available for this particular project, the presence of an IgG response in the UGT and ocular sites is a promising sign. However, the mucosal priming immune sites are generally thought to be mainly located in the Peyer's patches of the intestinal tract for an ocular response, whereas, they are generally thought to be located at distant sites (spleen and iliac lymph node) for a genital tract response [50]. The dominant Ig- subclass in the genital tract is IgG and it is thought that, at least in volume, IgG predominates over IgA [51]. IgG does however play a significant role at genital mucosal surfaces through exudation or translocation from plasma and also by being produced in the local B cells [52]. These unique characteristics of the immune system within the genital tract, and with *Chlamydia* itself targeting the mucosal epithelium, highlight the importance of producing both systemic and mucosal immune responses from the administration of a *Chlamydia* vaccine. In this current study, we demonstrated that the tri-adjuvant elicited systemic and mucosal humoral immune responses against *C. pecorum* MOMP vaccine antigen. The tri-adjuvant components are thought to act synergistically through different immune mechanisms [53]. The adjuvant and immune stimulating properties of poly I: C, polyphosphazine and HDP have been shown to have a synergistic effect in mouse and porcine models [43, 54]. The adjuvant combination of

CpG ODN, cationic host defense peptide (HDP) and PCEP resulted in the induction of a robust and long-lived immune response. Nonetheless, the combination of different adjuvants into a single formulation is an evolving approach to enhance the immunogenicity of the antigen, both in human and animal vaccine trials [53].

The protective immune response against the intracellular bacterium, *Chlamydia*, requires interferon gamma secreting CD4+ T cells [55] and neutralising antibodies in mucosal secretions [56]. Although the Th1 immune response is critical in chlamydial infections, the role of antibodies has been described in a considerable number of published articles in mouse and guinea pig models, and these support the role of antibodies in protective immunity [56-61]. One suggestion is that antibodies, in conjunction with T cell mediated adaptive changes, promotes an anti-chlamydial response through FcR mediated phagocytosis, complement activation and neutralisation [62]. Mucosal antibodies have been found to be associated with decreased shedding of *C. trachomatis* infections in women with genital tract diseases, such as cervicitis and uterine infections [63]. Mouse infection model studies, using B cell and Fc receptor deficient mice, also support the role of B cells in immune-protection [64]. Other studies in mice also suggest the immune-protective role of antibodies against secondary chlamydial infections [65]. However, antibody function ultimately depends on the cellular responses that regulate the adaptive changes at mucosal surfaces for genital tract [61].

Nonetheless, much of the information that has informed chlamydial vaccine development and disease progression is obtained from laboratory animal studies, particularly the mouse model. While there are significant differences between mice and koalas, the former is still considered to be the best experimental animal to elucidate the complex host-pathogen interactions [66].

Nevertheless, infection is usually self-limiting following a single challenge in mice, whereas in other animal models, including humans and koalas, chronic infections develop following undiagnosed and untreated conditions [55]. Moreover, mice are often treated with hormone injections (particularly progesterone), to synchronise the reproductive tract physiology and thus increase their susceptibility to infection [67]. Therefore, there are clearly significant host differences in immune response, including to immunisation, and these differences need to be taken in to account for vaccine development [68-70]. Prior to the work in this thesis, there was a relatively limited analysis of the humoral immune response to vaccine in koalas, except for the reports by Carey et al. [12] and Kollipara et al. [16] . The results presented in this thesis with koalas, identified the novel role of B cells specifically to elicit immune response through neutralisation, and might potentially contribute to the *C. trachomatis* human chlamydial vaccine design.

The surface exposed protein, MOMP, is a major immunodominant antigen on the surface of the chlamydial infectious elementary body. This protein consists of four variable domains interspersed with five constant domains. The high rate of substitution at the variable domains and the immunological pressure to mutate, promotes host immune evasion. Kollipara et al. previously reported that koala *C. pecorum* genotypes have high levels of diversity [13]. Despite the antigenic diversity of MOMP, our rMOMP vaccine was able to induce an antigen specific neutralising antibody mediated immune response. In addition, we were able to demonstrate specific antibodies titers against not only rMOMP, but also against native whole EBs, and this is an important characteristic for an effective vaccine.

The major aspect in analysing the protective humoral immune response induced by an effective vaccine is to demonstrate the functionality of the antibody response through neutralisation. Therefore, we performed a PepScan approach to better understand the epitope profile of the two different cohorts of koalas, namely diseased and vaccinated. The vaccinated animals showed a unique MOMP epitope profile, with 4 key epitope responses associated with neutralising ability against *in vitro C. pecorum* infection. A *C. trachomatis* vaccine study, which used a primate model, demonstrated that the neutralising antibodies produced following vaccination with native MOMP from either ocular or genital serovars, were highly serovar specific, even though there was only two amino acid differences between homologous and heterologous strains [18, 24]. In the mouse model, *C. trachomatis* serovar L1 vaccination through different routes, and subsequent *C. trachomatis* serovar F infection through the bursal route, induced limited protection against vaginal shedding, but no protection against infertility [71]. Interestingly, in our current study, the vaccine induced epitopes are all located in the conserved domains, perhaps explaining their role in cross-reactive neutralisation against diverse MOMP genotypes. While it has previously been assumed that antibodies must bind to externally presented epitopes to enable neutralisation [64], recent reports have shown that antibodies, via FcRn mediation, can internalize and neutralise virus [72] and intracellular bacteria such as *Chlamydia*, within the cytoplasm of epithelial cells [73]. Antibodies to internal proteins, such as the chlamydial NrdB, have also been shown previously to facilitate the neutralisation process [74]. In addition, during the infection process, the host immune system might contribute by releasing the previously inaccessible or unexposed protein from disintegrated cell walls, and thereby become exposed to B cell antigen receptors. Nonetheless, upon unfolding the native MOMP structure through limited proteolysis, the unexposed or conserved linear motifs can be exposed to the anti-

MOMP epitope B cell antibody [75]. The identification of common and hidden epitopes that induce cross-serovar protection poses a major challenge in several human pathogens, including hepatitis C virus, *Neisseria meningitides* and influenza virus [76-79]. The findings of our current study suggest that conserved and hidden B cell epitopes are poorly exposed to the immune systems in natural chlamydial infections, but that the rMOMP vaccine we evaluated, which presumably had only linear MOMP, has the ability to stimulate the immune system successfully and to elicit cross-reactive antibodies that can neutralise multiple serovars of *C. pecorum*. However, several vaccine studies have used the native form of MOMP, arguing that this could ideally represent and restore the EB's MOMP conformation into its original 3-D form, to elicit a more robust immune response [18]. However, this current koala vaccine study suggests that immunisation with recombinant linear MOMP is able to generate neutralising antibodies against epitopes in the conserved domain and might be capable of protecting against a wide range of *C. pecorum* infections in koalas. The previous koala vaccine study [15] demonstrated the cross-reactive properties of the immune plasma in either diseased or healthy animals, following vaccination. For example, koalas with *C. pecorum* strain F infection and subsequently vaccinated with heterologous rMOMP of *C. pecorum* were still able to neutralise *C. pecorum* infection [15]. The results of our current study confirm that koalas vaccinated with non-conformational rMOMP of *C. pecorum*, can develop an antibody response that can effectively neutralise homologous as well as heterologous infection [15]. A recent study in a mouse model of *C. trachomatis* infection, showed that a multivalent peptide vaccine was able to induce neutralising antibodies directed to the variable domain of the MOMP protein [57]. Furthermore, their study suggests that a small fragment of the MOMP protein was able to induce specific and neutralising *C. trachomatis*-specific antibodies. This study further confirms the immune protective role of

antibodies in challenge infection. Nonetheless, vaccine induced antibodies were able to reduce the bacterial burden and prevent the upper reproductive tract pathology in subsequent challenge infection [57]. Similarly, a recombinant epitope based vaccine strategy has been successfully demonstrated by Murtada et al.[80]. They showed that this vaccine can stimulate antigen specific systemic and mucosal antibody responses [80]. In a recent mouse study, these authors reported that rMOMP adjuvanted with ISC can reduce the infection burden and prevent subsequent oviduct pathology [81]. This study indicated that the vaccinated animals produced different cytokines and chemokines, specially, IFN-γ, TNF-α, at the genital mucosa. There was an increased accumulation of macrophages and neutrophils at this site. One of the major findings of this study was that antibodies generated following vaccination might contribute towards immune protection through enhancing innate and adaptive responses [81].

In addition to the testing of rMOMP protein vaccine in captive koalas at the Lone Pine Koala Sanctuary, this thesis work also examined chlamydial vaccination of wild koalas in a population in South East Queensland, termed the MBRL population. This larger population contained around 300 koalas and 60 koalas in this population were used for chlamydial vaccination studies. Of these 60 koalas, half of the animals were assigned to an unvaccinated control group, to evaluate the prophylactic and therapeutic effect of the current version of the koala-*Chlamydia* vaccine. While systemic antimicrobial drugs are used for treating chlamydial infections, several studies have shown the ineffectiveness of the current antibiotic regimes for controlling severe chlamydiosis in koalas [10, 82]. As koala chlamydial infection is widespread and often asymptomatic in free ranging animals, a prophylactic vaccine is of paramount importance. Recent studies showed that women with *C. trachomatis* infection

without clinical disease, and treated with antibiotic, had a significant level of subsequent re-infections within a 1 year time period [83]. This clearly suggests that some individuals receiving antibiotic treatment did not develop protective immunity [84]. Similarly, molecular studies have suggested similar observations with wild koalas without signs of clinical chlamydiosis [5]. Therefore, we evaluated the vaccine induced immune response within this cohort of naturally infected koalas. Perhaps not surprisingly, we found higher antibody responses in vaccinated koalas which had a current chlamydial infection. Notably, the level of neutralising antibodies was significantly higher in the vaccinated group compared to control koalas. However, we found a strong and statistically significant correlation between higher infection load and lower antibody titre. This might be an interesting finding as koalas with a high infectious loads at genital and ocular mucosal sites were most likely to have infection with clinical chlamydiosis [85].

A part of the thesis work utilised this wild koala population to study, (a) immune response (antibodies) to natural infections as well as the (b) immune response following vaccination. An important finding in this study was that a different and unique set of antibodies (to different epitopes on MOMP) were induced by vaccination, compared to those seen in natural, live infections. All vaccinated koalas produced neutralising antibodies to 4 key epitopes that were located in the conserved regions of MOMP. This is promising for vaccine development because these epitopes would be conserved across the multiple MOMP genotypes. Not only were the vaccine induced antibodies unique, but they were neutralising in our *in vitro* assay. By comparison, the antibodies present in sera of naturally infected koalas with clinical disease, were found not to have any neutralisation ability. Although it has previously been thought that antibodies predominantly bind to the surface exposed epitopes

to enable the neutralisation [86], we found that antibodies targeting apparently internal epitopes, such as those in the conserved/membrane anchored regions of MOMP, could also effectively neutralise infectivity. One possible explanation for this is that, recent studies have shown that antibodies can be taken up inside the epithelial cells through FcRn mediated transport and thereby neutralise virus [72] and intracellular bacteria, like *Chlamydia* [73].

The final aspect of this thesis work was to compare the responses of two MOMP vaccines, containing the same recombinant MOMP proteins, but mixed with two different adjuvants (Chapter Six). Overall, both adjuvants induced a strong cell mediated immune response through IFN-γ mRNA expression in circulating PBMCs. The animals in both cohorts given these two vaccines showed a very specific and distinct neutralising antibody profile, though the neutralisation level was generally similar. It is generally thought, IFN-γ secreting CD4+ T cells and neutralising antibodies at the infection site provided immune protection against chlamydial infection [63].

In several koala *Chlamydia* vaccine trials, our group measured the proliferative response of the PBMCs against rMOMP protein or UV-inactivated EBs of *C. pecorum* [14, 15, 87]. These studies were unable to measure the cytokines profile following vaccination because koala specific cytokine assays were not available at the time of these earlier studies. Cytokines are the immune system's molecules that modulate the immune response against chlamydial infection or vaccination. In this current study we first measured the koala specific mRNA expression of a selected Th1 cytokine, namely IFN-γ, in circulating PBMC following vaccination. For an intracellular organism like *Chlamydia*, the host immune system requires a CD4+ T cell response for the production of a protective immune response, as shown in

several animal models [88, 89]. CD4+ T cells are classified into two cohorts based on their type of cytokine production, T helper 1 (Th1) and T helper 2 (Th2) cells. Th1 cells produce IFN-γ and IL-12 cytokines to regulate immunity against intracellular infections, whereas, IL-4, IL-5 and IL-13 are the dominant cytokines for the mediation of humoral immunity against parasite infections [90]. The IFN-γ response has been shown to be critical for protection against chlamydial infection in the mouse model [91, 92]. However, our current study only involved clinically healthy koalas that presented with no sign of chlamydial infection. While we found significant IL-17A gene expression in some koalas from both cohorts, the role of IL-17A in protection versus disease is still unclear [93]. It is interesting that both IL-17A and IFN-γ have been implicated in pathological damage in the mouse model [94]. Recent studies have shown a strong correlation between higher expression of IL-17A with clinical chlamydiosis and cervicitis in koalas [93] and women [95]. Even though the role of IL-17A in chlamydial pathogenesis has yet to be fully understood, our study demonstrated that vaccination induced expression of this cytokine at variable levels in koalas. IL-17A is a strong recruiter of neutrophils [96] which secrete anti-microbial peptides namely defensins and cathelicidins, at the infective sites [97] and which play a critical role in host defense against infection [98]. These neutrophil-derived defensins have significant immuno adjuvant properties, promoting an antigen specific Ig response in mouse model. In addition, defensins induced an increased production of IFN-γ, a Th1 cytokine [99]. Recent studies identified that human defensins as a potent ligand for the chemokine receptor (CCR6), which is present on dendritic as well as T cells, thereby, promoting a link between the innate and adaptive immune responses [100]. Tuberculosis vaccination in a mouse model elicited IL-17 producing CD4+ T cells which promoted the production of chemokines that recruit the CD4+ T cell producing IFN-γ, eventually limiting the bacterial growth [101]. Moreover, both these

cytokines have shown immune protection via the up regulation of the inducible nitric oxide synthase (iNOS) and nitric oxide (NO) production against *Chlamydia muridarum* [102]. We did not find any measurable expression for the IL-10 and TNF-α mRNA in our koala vaccine study, and their role in chlamydial infection and immunity remains controversial. Generally, IL-10 suppresses the secretion of pro-inflammatory cytokines [103] and has been linked to chronic infection in the mouse model [104]. Similarly, higher expression of this cytokine has been attributed to tubal pathology [105] and infertility [106, 107] in women. While several of the koalas analysed in our study, that had an ongoing chlamydial infection, had variable IL-10 and TNF-α expression [93, 108], the latter cytokine has been associated with decreased chlamydial shedding with co-expression with IFN-γ in vaccinated mice [109]. In these mouse model studies, it was shown that TNF-α produced by CD8+ T cells, could promote inflammation in the oviduct following *C. muridarum* infection [110] but that CD4+ T cells producing IFN-γ and TNF-α are generally immune-protective. A recent study by Mathew et al. [111] using koalas with natural infection and disease showed significantly higher IFN-γ expression compared to clinically healthy individuals [93] and this over amplified cytokine level might hinder the role of T cell response in preventing future infections [55] and dissemination of the pathogen [68, 112]. Our results therefore are very promising for the ability of both vaccines to elicited relevant immune response in koalas against chlamydiosis.

While there were many similarities between the two groups of koalas vaccinated with the different adjuvants, there were also some key and very interesting differences. The animals showed comparatively similar immune responses regardless of the two different adjuvant regimes. The total antibody titers were marginally higher in the ISC animals, but showed no difference in terms of neutralisation percentage. Both ISC and single dose tri-adjuvant

formulations induced high antibody titers with good *in vitro* neutralisation levels. This strongly suggests the feasibility of the single dose vaccine to elicit functional antibody response to neutralise the infectious EBs in free ranging koalas. Furthermore, vaccinated koala PBMCs expressed promising levels of IFN-γ and IL-17A in response to stimulation with UV-inactivated *C. pecorum* infection. Vaccine studies in non-human primates for HPV and malaria infection have shown that this tri-adjuvant component is able to induce antigen specific antibodies and is a potent inducer of IFN-γ from the PBMCs [30, 113]. In addition, we are using koalas which are out-bred animals, with diverse MHC-II molecules across their range especially in the northern state of Queensland and New South Wales [114, 115]. This genetic variation may have an effect on infections and disease severity across the koala's range, but importantly, it could also impact on the vaccine response.

The results of this thesis have demonstrated that a recombinant *C.pecorum* protein, in combination with a novel tri-adjuvant, can produce a strong antibody and CMI response following a single immunisation in koalas. The resultant antibody response was not only high titer but was also directed against several conserved MOMP epitopes. This characteristic is potentially promising for protection against a wide range of koala *C. pecorum* strains. A vaccine formulated with multiple MOMP types elicited neutralising and antigen specific antibody. Perhaps, even more promising was the IFN-γ (*C. pecorum* specific) response induced by vaccination.

The current rMOMP vaccine has shown promising immune response either in combination with ISC or Tri-Adj. We did not observe any distinct variation in these two adjuvant regimes, apart from epitopes specificity. Considering the multiple doses ISC, Tri-Adj looks very

promising. Furthermore, the Tri-Adj induces extra epitope specificity though its neutralising ability was not increased. It will be interesting to measure the actual concentration of the specific epitopes in the systemic circulation following vaccination or natural infection. Nonetheless, we evaluate the Tri-Adj in a koala population that shown the evidence of the therapeutic effect and looks quite promising (manuscript in prep). We currently able to measure the IgA antibody response in secretions. Therefore this immune markers will enhance the detection of specific immune response following vaccination. The most important aspect of this vaccine trial was that the rMOMP vaccine was not aggravate any pathological response. We evaluate this vaccine in naturally infected koalas as well as the non-infected animals.

In summary, our data from this project has demonstrated several key findings including, (a) the single dose tri-adjuvant, combined with rec MOMP protein, can elicit a long lasting (54 weeks) cellular and humoral immune response in koalas, (b) recombinant MOMP based vaccines elicit a specific and distinct humoral immune response with neutralising antibodies (c) the prototype rMOMP protein vaccine is able to induce a Th1 biased immune response and has the potential to be used as therapeutic and prophylactic treatment of wild koalas.

Future directions

The study provides the first experiment that utilised tri-adjuvant components in koalas and we evaluated the rMOMP based protein vaccine in the natural host in its wildlife setting. While the progress looks promising, there are still several issues that need to be addressed before any koala Chlamydia vaccine can be widely used.

The studies presented in this thesis have successfully evaluated a rMOMP based protein vaccine in captive koalas as well as in wild koalas. In addition, it would be ideal to test the protective efficacy of the vaccine in several wild koala populations. Though, we measured the prophylactic effect of the vaccine, the number of organism recovered at fixed time points following infection could be used to define the severity of the infection or vaccine response. This is significantly important to measure the vaccine efficacy to determine whether a vaccine administered can protect against either infection or disease.

The diverse immunological pathways that regulate the development of the immune responses are mediated through distinct immuno-regulatory cytokines produced by two types of regulatory T cells (CD8+ and CD4+ T cells). In the process of koala *Chlamydia* vaccine development, it is critical to identify the relevant immunological pathways in naturally infected animals as well as in vaccinated animals. Recent identification of the CD4+ T cell in koala [116] is a promising aspect to measure this T cell subset in immunised animals. In this thesis, we looked at the systemic cytokine response patterns of vaccinated animals. However, cytokines generally have a localised immune response during an infection or following vaccination. Nevertheless, the pathology produced by *C.pecorum* is also localised mainly in the reproductive site. However, looking at the cytokine profile at the urogenital and ocular site could provide a more complete picture of the disease pathogenesis and better inform vaccine design. The cytokine profiles measured during this thesis measured mRNA expressions, not actual protein. However, the majority of the cytokines are regulated through post-transcriptional modifications and might not truly reflect the mRNA expression within the biologically active protein molecule. This post-transcriptional control mechanism that affects the final expression of the protein might be an issue to address in future study.

The two immunoglobulin's subclasses, IgG and IgA, presumably play a critical role in the koala immune process. Both these antibodies are transported via FcRn and PlgR receptors respectively and the expression of receptors has been shown to vary among different cell types and anatomical location in animal and human studies [117, 118]. It has been reported that IgA provides protection in the infection site at the very initial stage of the infection [119]. While the role of IgA against primary or secondary chlamydial infections appears to be negligible [120], the role of this antibody subclass is strongly correlated with a reduction of chlamydial burden in the male prostate gland [121].Therefore, it is important to measure this immunoglobulin subclass in the mucosal secretions during the infection process or following vaccination. More importantly, it will be critical to measure the neutralisation ability of this antibody along with IgG.

Investigations in relation to the genetic diversity on the koala's immune systems are yet to be done. Studies have shown that a minimum level of genetic diversity exists among certain island koala population (French and Kangaroo populations) [122]. Indeed, these koala populations have been reported as *Chlamydia* free isolated populations, though low genetic diversity might suggest a link to enhanced disease susceptibility [123]. However, large scale studies utilising the highly variable coding genes such as MHC class II molecules [124] need to be done to better understand the contribution of genetic diversity to disease biology. Another important confounding factor which might affect the koala's immune system response to chlamydial infection is the presence of KoRV infection [125]. Studies have shown a higher prevalence of KoRV infection in the southern koalas. This study however failed to establish a link between KoRV RNA expression levels and chlamydiosis [126].

Therefore, this aspect on the immune systems might be an important issue to investigate in future studies.

While the vaccine research to date looks promising, a live challenge infection in a *Chlamydia* koala model is essential in the future. However, from the ethical point of view, this seems to be a challenge to implement. In addition, determining the age of vaccination would be a major issue in koala-*Chlamydia* vaccine design. We have observed high level of variation for animal-to-animal immune responses following vaccination. Future studies should aim to minimise these variations, either by the use of age, sex match cohorts or to better understand the MHC restriction of the MOMP epitopes. A recent study with mathematical simulation suggests that, female koalas at 1-2 years of age would be the preferred target population cohort for achieving optimum results following vaccination [127]. This period is very critical for the female koalas, as they attained sexual maturity at this stage. The development of immunity at this time point would potentially reduce the transmission of chlamydial infection. Recent studies in a mouse model suggests the significant role of immunised male or female animals in subsequent disease transmission [81]. Additionally, few studies have focussed on the significant role of male koalas in disease transmission pose to critically address this gender issue in vaccine formulations.

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