**Vaccination of healthy and diseased koalas *(Phascolarctos cinereus)* with a *Chlamydia pecorum* multi-subunit vaccine: evaluation of immunity and pathology**

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**Abbreviations:**

MOMP – Major Outer Membrane Protein

NrdB – Ribonucleotide reductase small chain protein

**Abstract**

Chlamydial infections represent a major threat to the long term survival of the koala. Antibiotics are not a viable option for control of infections in wild animals and a successful vaccine would provide a valuable management tool. Vaccination however has the potential to enhance inflammatory disease in animals exposed to a natural infection prior to vaccination, something that was observed in early human and primate trials of whole cell vaccines to prevent trachoma. In the present study, we vaccinated both healthy koalas as well as clinically diseased koalas with a multi-subunit vaccine consisting of *C. pecorum* MOMP and NrdB mixed with immune stimulating complex as adjuvant. Following vaccination, there was no increase in inflammatory pathology in animals previously infected with *Chlamydia*. Strong antibody (including neutralizing antibodies) and lymphocyte proliferation responses were recorded in all vaccinated koalas, both healthy and clinically diseased. Vaccine induced IgG specific for both vaccine antigens were observed not only in plasma but also in ocular secretions. Our data shows that an experimental chlamydial vaccine is safe to use in previously infected koalas, in that it does not increase infection-associated pathology. Furthermore, the prototype vaccine is effective, as demonstrated by strong levels of neutralizing antibody and lymphocyte proliferation responses in both healthy and clinically diseased koalas. Collectively, this work illustrates the feasibility of developing a safe and effective *Chlamydia* vaccine as a tool for management of disease in wild koalas.

Keywords:

## Introduction

The koala (*Phascolarctos cinereus*) is a native Australian arboreal marsupial and the only surviving member of the family, *Phascolarctidae*. Despite its position as an icon of the unique biodiversity in this country, wild koala populations in geographically dispersed regions throughout Australia continue to experience serious declines in numbers, leading to localised extinctions. The rapidity of this decline can be attributed to anthropogenic influences [1] such as (i) land clearing and loss of habitat, resulting in the fragmentation of koala colonies [2]; (ii) motor vehicle trauma [3]; and (iii) dog attacks [1]. Disease is also a major cause of mortality in the koala. Indeed, a recent modelling study showed that targeting disease, among the many variables adversely affecting koala survival would have the greatest potential impact on stabilising population decline [4].

The main aetiological agents of disease in the koala belong to the family *Chlamydiaceae*, obligate intracellular bacteria and pathogens of humans and animals. *Chlamydia pneumoniae* is a cause of atypical pneumonia in humans [5] and is associated with disease in a range of animal host species including the koala [6]. *Chlamydia pecorum* is primarily an animal pathogen and a cause of disease in cattle, sheep, pigs and wildlife such as the koala [7]. *C. pneumoniae* and *C. pecorum* have been detected in most wild koala populations screened with *C. pecorum* found to be significantly more widespread and the more pathogenic of the two species [8-9]. In the koala host, chlamydial infections have been associated with a spectrum of diseases ranging from ocular disease leading to blindness, rhinitis and pneumonia, as well as urinary and genital tract disease, resulting from inflammation and fibrosis of the bladder and the upper female genital tract [10]. Whilst human chlamydial infections can be treated with antibiotics, this is not a preferred option available for eliminating disease in wild koala populations. An effective vaccine to prevent the adverse consequences of chlamydial infections in koalas would provide an important management tool to stop the population decline in this species.

MOMP encoded by the *omp*A gene and conserved across the members of the *Chlamydiaceae*, is a highly immunogenic protein and the leading vaccine antigen candidate for both human and animal chlamydial vaccines. Despite this, vaccination with MOMP does have its challenges; primary amongst these is that the surface-exposed variable domains of

human *C. trachomatis* MOMP are genetically diverse, as a consequence of immune selection. As such, in human *C. trachomatis* vaccination models, immunization with MOMP from one serovar will only elicit protection against the same and closely related serovars [11]. A possible solution to this problem would be to include other antigens that are conserved across serovars/genotypes of *C. trachomatis* (or *C. pecorum*), together with MOMP, in a multi-subunit vaccine. This approach has been shown in a number of studies using the mouse model to provide enhanced protection against infection with *C. muridarum* or *C. trachomatis* and in some studies this approach even provided protection against heterologous serovars [12-14].

In a first step to evaluate the feasibility of a chlamydial vaccine in the koala, our group recently evaluated the immunological response of healthy koalas to a multi-subunit chlamydial vaccine, derived from non-koala chlamydial species [15]. In contrast to earlier reports suggesting that the koala was 'immunologically lazy' [16], we observed high levels of antigen-specific peripheral blood mononuclear cell (PBMC) proliferation (lasting >1 year) and sustained plasma antibody levels against three antigens, including MOMP, in immunized animals. Importantly, koala IgG antibodies were able to inhibit in vitro infection of McCoy cells with *C. muridarum* (80-90% inhibition at a 1:10 dilution), the strain from which MOMP was derived and, also partially neutralized *in vitro* infectivity of a koala strain of *C. pneumoniae* (30-40%), suggesting some cross-reactivity with the *C. pneumoniae* species.

While these results are very encouraging, the study was performed in healthy, *Chlamydia*-free koalas and utilized chlamydial antigens from chlamydial species that do not infect koalas. Therefore, questions remain about (1) the safety and immunological response to vaccination in animals with evidence of a previous or ongoing chlamydial infection and (2) the choice of the vaccine antigens themselves. In regards to the latter question, the main antigen used in our previous trial was MOMP. Molecular analyses of koala *C. pecorum* MOMP-encoding *ompA* gene sequences from geographically distinct wild koala populations has revealed extensive sequence diversity (10 – 30%) amongst the surface-exposed variable domains of this protein [9,17,18], similarly to that seen for *C. trachomatis* serovars [19]. Because of this diversity it is unlikely that a vaccine based on a single *C. pecorum* MOMP would provide protection across the genetically diverse and distinct *C. pecorum* strains

detected in wild koalas. A possible solution to this problem is the addition of MOMP from other *C. pecorum* strains or other antigens that are conserved across multiple koala isolates.

In the present study, to address these important questions, we immunized healthy koalas as well as koalas with clinically significant ocular or urogenital tract disease with a multi-subunit vaccine containing a single koala *C. pecorum* MOMP genotype together with NrdB, an antigen highly conserved across all chlamydial species. By closely monitoring the (i) clinical health of the animals, (ii) the antibody-based immunological response of vaccinated animals, (iii) the cross-reactivity of koala antibodies to different MOMP genotypes, and (iv) lymphocyte proliferation against vaccine candidates, we show that koala vaccination is safe and induces an immune response that may provide ongoing protection from natural *C. pecorum* infections in the future.

## Materials and Methods

### Koala *C. pecorum* strains used in the study

Four strains of koala *C. pecorum* were used in this study. All strains came from wild koala populations in South East Queensland, Australia that were sampled as part of other studies [18]. Swab samples were collected from either the urogenital tract or ocular site of koalas showing clinical signs of chlamydial disease and were tested by a *C. pecorum*-specific PCR assay [20]. Genomic DNA from extracted from positive *C. pecorum* swab samples was amplified by a PCR that targets the full-length *omp*A gene. The products were sequenced and then assigned an *omp*A genotype. For this purpose, *omp*A genotypes originally designated in [17], were used as the starting point. For newly detected *C. pecorum* strains, an *ompA* sequence that had more than 1% base pair differences to previously designated genotypes was assigned a new genotype. The four strains that were selected for this study represent different *omp*A genotypes; genotype A (Ipswich/Tabi/LE; geographical location/koala name/left eye), genotype F (Nar/DeeDee/UGT), genotype G (Mt.Cotton/MarsBar/UGT), genotype H (Coomera/Ned/UGT).

### Production of recombinant MOMP and NrdB proteins for vaccination

*E. coli* strains JM109 and BL21 (DE3) pLysS competent cells were used for molecular cloning and protein expression and purification, respectively. *E. coli* strains transformed with the respective expression constructs were grown in Luria-Bertani broth (LB; 10 g tryptone, 5 g yeast extract, 10 g NaCl per L), containing 75 ug/ml ampicillin, at 37°C with constant shaking. *E. coli* cell growth in liquid media was assessed by measuring $OD_{600}$.

Koala *C. pecorum* NrdB and four genetically distinct koala *C. pecorum* MOMPs (A, F, G and H) were PCR-amplified, cloned, expressed and purified for use in the vaccine trial. The full-length *nrdB* gene was PCR amplified using primers NrdBXhoI (5'–TTTTCTCGAGAAAGCAGATATTCTCAGTGG-3') and NrdBKpnI (5'–TTTTGGTACCTTACCAACTCAAACTTGCCG – 3') and Vent DNA polymerase (New England Bioscience) from *C. pecorum* genotype G (Mars Bar isolate) DNA template. Truncated *ompA* genes for each *C. pecorum* MOMP genotyped strain, excluding the N-terminal signal peptide sequence, were similarly amplified using primers OmpAXhoI (5'–

AAAAACTCGAGTTGCCTGTAGGGAACCC–3') and OmpAKpnIn (5' – AAAAAGGTACCTTAGAATCTGCATTGAGCAG – 3'). Each PCR product was amplified to generate PCR products with 5' – *Xho*I and 3' – *Kpn*I restriction sites and were ligated into a N-terminal polyhistidine (His) pRSET-A expression vector (Invitrogen), following *Xho*I/*Kpn*I double digestion, which was similarly digested. His-tag expression constructs were transformed into BL21 (DE3) pLysS competent *E. coli* cells and grown in LB media with 100 ug/mL ampicillin at 37°C. At an $OD_{600}$ of 0.4 – 0.6, His-tagged protein expression was induced by the addition of 1 mM IPTG for 4 h. Cells expressing recombinant *C. pecorum* MOMP were harvested by centrifugation and resuspended in lysis buffer I (50 mM phosphate buffer pH 7.0, 8M urea, 2 mM PMSF, 50 uL Protease Inhibitor Cocktail (Sigma) and 1 mg/mL lysozyme). Lysed cells were incubated with TALON metal affinity resin (Clontech) at 4 °C for 1 h with gentle mixing. Resin was then repeatedly washed with wash buffer I (50 mM phosphate buffer pH 7.0, 300 mM NaCl, and 8M urea) and protein was eluted with elution buffer I (50 mM phosphate buffer pH 7.0, 300 mM NaCl, 150 mM imidazole and 8M urea) by centrifugation.

Cells expressing recombinant *C. pecorum* NrdB were harvested by centrifugation and resuspended in lysis buffer II (50 mM phosphate buffer pH 7.0, 2 mM PMSF, 50 uL Protease Inhibitor Cocktail (Sigma) and 1 mg/mL lysozyme) and lysed by repeated freeze/thawing. Lysed cells were incubated with TALON resin at 4 °C overnight with gentle mixing. Resin was repeatedly washed by gravity-flow with washing buffer II (50 mM phosphate buffer pH 7.0, 500 mM NaCl, 5 mM imidazole and 0.1 % Triton X-100) before being eluted with elution buffer II (50 mM phosphate buffer pH 7.0, 500 mM NaCl, 150 mM imidazole).

Purified protein samples were dialysed into PBS and recombinant protein yield determined by the Bicinchoninic Acid (Thermo Fisher Scientific, Australia) method using an ovalbumin standard curve as a control. Purified protein samples were resolved on 12 % SDS-PAGE gels and detected following Coomassie blue staining and Western blotting (after transfer to nitrocellulose) with a monoclonal anti-His antibody (Sigma-Aldrich, Castle Hill, Australia). Purified recombinant *C. pecorum* proteins were used for (a) koala immunizations, (b) ELISA assays, (c) Western blot assays and lymphocyte proliferation assays.

Experimental groups of koalas and immunization schedule

22 wild koalas, which presented to the Australia Zoo Wildlife Hospital (Beerwah, Queensland) for treatment of a variety of afflictions, were used for this study. The koalas were assigned into two groups. Group A (A1, A2 and A3) were free of chlamydial disease and negative to *C. pecorum* - specific 16S rRNA quantitative PCR (qPCR) and serological assays. Group B animals (B1 and B2) showed clinical evidence of classic chlamydial disease and positive results to our *C. pecorum*-specific 16S rRNA qPCR and serological tests. For the purposes of this study, the classic chlamydial disease signs were kerato-conjunctivitis and/or cytological or sonographic evidence of urogenital tract disease (prostatitis [males] and metritis/ovarian bursitis [females] and/or cystitis [both sexes]). After admission to the wildlife hospital, each koala received a thorough veterinary health assessment and was treated for their respective conditions. Koalas were only recruited into the vaccine trial on completion of treatment. Koalas with chlamydiosis were treated with parenteral chloramphenicol and adjunctive therapies as prescribed by the consulting veterinarian. At the time of recruitment into the trial, the koalas were deemed by the respective consulting veterinarians to have completed their treatment and be suitable for release.

The 18 koalas recruited for the main trial (given three doses of either vaccine or placebo) were divided into 4 groups (Table 1): (A1) No detectable clinical signs of chlamydial disease on admission to hospital and receiving the vaccine (4 koalas); (A2) No clinical disease on admission but receiving the placebo (2 koalas); (B1) Showing signs of clinical disease on admission and receiving the vaccine (8 koalas); (B2) Showing signs of clinical disease on admission but receiving the placebo (4 koalas).

In a parallel sub-study we compared immunization of healthy animals with three doses (above animals, group A1) to immunization with two doses of vaccine. These additional four koalas were assigned to group A3 and had no clinical disease on admission.

Animals receiving three subcutaneous immunizations were vaccinated on days 0, 28 and 56. The two dose group received subcutaneous immunizations on days 0 and 56. Each dose of vaccine consisted of 50 µg of recombinant genotype G MOMP protein, 50 µg of recombinant G NrdB protein mixed with 50 µg Immunostimulating Complex (ISC) in 0.5 mL PBS. Animals in placebo groups received ISC alone.

Sample collection

Each of the koalas was anaesthetised for veterinary assessment and samples collected on day zero, and then at monthly intervals five additional times. A 10 ml sample of blood was collected into a blood tube containing the anticoagulant lithium heparin. The blood samples were stored at 4 °C until further processing occurred later in the day. Swabs were collected from each of the following sites using aluminium-shafted cotton-tipped swabs (Copan; Interpath Services, Melbourne): left conjunctiva, right conjunctiva, nasal cavity and urogenital sinus (females) or urethra (males).

Scoring of clinical disease

During general anaesthesia at each sampling event, each koala was also subjected to a thorough veterinary health assessment, which included sonographic examination of the urinary and reproductive tracts, close examination of the eyes, and urinalysis, including microscopic examination of urine sediment. Clinical assessment included measurement of packed cell volume (PCV) and total plasma protein (TPP) and microscopic examination of a Diff Quick stained blood smear.

Chlamydial disease signs present at ocular and urogenital tract sites were scored for each koala using specific criteria. Scoring for ocular and urogenital tract was done on a 0 – 3 scale. At the ocular site, koalas with no detectable disease were assigned as 0; 1 - animals with acute/sub-acute kerato-conjunctivitis; 2 – koalas with chronic kerato-conjunctivitis without evidence of disease; 3 – koalas with chronic, active kerato-conjunctivitis, characterised by active disease. At urogenital tract, animals with no detectable disease were assigned 0; 1 – koalas with acute or sub-acute cystitis; 2 – chronic, inactive cystitis and; 3 – koalas with chronic, active cystitis. To ensure consistency, all clinical assessments were carried out by the same experienced veterinary staff. At the time of clinical assessments, veterinary staff was kept blind regarding the groupings of the animals into vaccine or placebo groups.

Prior to recovery from anaesthesia, vaccinations or placebos were administered by subcutaneous injection over the right thorax caudal to the right elbow. Following

vaccination, koalas were monitored closely by hospital staff for signs of swelling at vaccination sites or systemic reactions to vaccination for 48 hours post vaccination.

Determination of *C. pecorum* infection status in vaccine trial animals

Apart from the scoring system as mentioned above, we have assessed the disease status with *C. pecorum* 16S rRNA gene specific qPCR [20] on pre-immunized swabs and MOMP immune blots using preimmunized sera from all the animals in the trial.

Measurement of anti-MOMP and anti-NrdB antibody responses

Enzyme-linked immunosorbent assay (ELISA)

96 well ELISA plates (Greiner bio one medium binding; Interpath, West Heidelberg, Australia) were coated with recombinant *C. pecorum* MOMP G (2ug/well) and *C. pecorum* NrdB (2ug/well) proteins, respectively in Borate-buffered saline (pH = 8.4) and incubated for 2 hrs at 37 °C. Post incubation, wells were washed 3x with PBS-T and then blocked in 5% skim milk in PBS-T for 1 hr at 37 ˚C. Plasma samples, serially diluted two fold in PBS-T on a separate plate, were added to the test wells (50 µl/well). All plasma samples were initially diluted to 1/500, except for *Chlamydia* infected samples, which were diluted to 1/1000. All the mucosal secretions (urogenital tract and conjunctival sites) were initially diluted to 1/20. Plates were then washed 4x with PBS-T and then incubated with sheep anti- Koala IgG [15] (50 µl/well) and incubated for a further 1 hr at 37 °C. Finally, after four washes, HRP-labelled rabbit anti-sheep IgG (1:1000 Southern Biotech/Invitro Technologies, Cleveland, Australia) was added (50 µl/well) to the wells and incubated for 1 hr at 37 ˚C. After five washes with PBS, 50 µl/well of TMB substrate (50 µl TMB in 5 ml of PCB) was added and incubated for 5 min at room temperature. The HRP-TMB reaction was stopped by adding 50 µl/well of 1 M $H_2SO_4$. The optical density was then read at 450 nm (Bio-Rad, North Ryde, Australia). End point titers of all the samples were defined as OD values equivalent to the mean plus two times the standard deviation of the negative controls.

Western blot

Western blots were performed with recombinant *C. pecorum* His-tagged MOMP A, F, G and H as antigens. 5 µg of each of the antigens were loaded onto 0.75 mm wide 12 % SDS-PAGE gels (110 V for 1 hr). Following transfer onto nitrocellulose membranes (Pall Corporation) at 90 V for 1 hr, they were blocked for non-specific binding in blocking buffer (5 % Skim milk in 1xTBS) overnight at 4 ˚C or for 2 hrs at room temperature. Post blocking, plasma samples were added on to the membrane at 1:1000 dilutions in blocking buffer and incubated overnight at 4 ˚C or 2 hrs at room temperature. Membranes were then washed 4x with 1xTBS-T for 5 min each. Secondary antibody (anti-koala IgG raised in sheep) was added at 1:1000 dilution in blocking buffer and incubated for 1 hr at room temperature. Membranes were again washed 4x with 1xTBS-T for 5 min each. Finally, tertiary antibody (anti sheep IgG-HRP raised in rabbit; Southern Biotech/ Invitro Technologies, Cleveland, Australia) was added onto the membranes at 1:1000 in blocking buffer for 1 hr at room temperature. Membranes were then washed 5x with 1xTBS-T for 5 min each. Blots were visualised for bands by adding Enzymatic Chemiluminescence substrate (Thermo Fisher Scientific, Australia).

*In vitro* neutralization assay

HEp-2 cells were seeded in 48 well plates (Greiner bio one; Interpath, West Heidelberg, Australia) and grown overnight until 70-80 % confluent. *C. pecorum* genotype G purified EBs (50,000 IFU) were added to the plasma samples (diluted 1:10 in cell culture medium) and incubated for 1 hr at 37 ˚C under 5% $CO_2$. Medium was removed from the HEp-2 monolayers and replaced with plasma samples and incubated for 4 hrs. Post incubation, samples were replaced with fresh media containing 1 µg/ml cycloheximide (Sigma-Aldrich, Castle Hill, Australia). Cells were further incubated for 48 hrs and then fixed with 100 % methanol. Monolayers were initially stained with anti-chlamydial EB raised in rabbit for 1 hr followed by washing and then incubated with goat anti-rabbit IgG BIOT (Southern Biotech/ Invitro Technologies, Cleveland, Australia). Wells were washed with 4x TBS-T and then stained with Strepatavidin-HRP (Southern Biotech/ Invitro Technologies, Cleveland, Australia). Finally, the monolayers were stained with 3, 3'-Diaminobenzidine (DAB; Thermo Fisher Scientific, Australia), prepared in peroxide substrate buffer. Both cells and inclusions were counted

under the microscope and a mean of 10 fields of view for each well were counted and the neutralization percentage determined compared to media only controls.

Measurement of anti-MOMP and anti-NrdB lymphocyte proliferation response

Blood samples were centrifuged at 233x***g*** (Beckman Coulter Allegra X-15R) for 5 min. Plasma was frozen for further studies at -80 ˚C. 10 ml of 1x PBS was used to dilute and resuspend the blood cells, which were mixed thoroughly. The cell suspension was then carefully layered onto a 10 ml Ficoll-Paque$^{TM}$ PREMIUM (GE Healthcare, Rydalmere, Sydney) and centrifuged at 650x***g*** for 25 min with the brake off. PBMC's were isolated from the buffy coat interface and suspended in a fresh tube containing 20 ml 1x PBS. Cells were further washed two times with 1x PBS at 400×***g*** for 10 min and counted. Cells were suspended at $2x10^6$ cells/ml in PBS containing 5 % fetal calf serum. Carboxyfluorescein succinimidyl ester (CFSE; Sigma-Aldrich, Castle Hill, Australia) was used at 5 µM to label the cells, which were incubated for 5 min in the dark. Cells were then washed two times with cold 1x PBS containing 5 % fetal calf serum (Southern Biotech/ Invitro Technologies, Cleveland, Australia) to quench the excess dye. Finally, the cell pellet was suspended in culture medium (RPMI 1640 containing 5 % fetal calf plasma, 2 mM L-glutamine, 10 mg/ml streptomycin sulphate, 2 µg /ml gentamycin and 50 µM 2-β-mercaptoethanol). A 1:1 mixture of cells ($2x10^5$) and antigens (8 µg of *C. pecorum* recombinant MOMP and recombinant NrdB) were added into 96 well flat bottom plates (In Vitro Technologies, Noble Park North, Australia). Concanavalin A (Sigma-Aldrich, Castle Hill, Australia) at a concentration of 2.5 µg/ml was used as a positive control and culture media with labelled cells were used as negative controls. Plates were then incubated for 5 days at 37 ˚C in 5% $CO_2$. After 5 days, cells were washed in 1x PBS and then fixed in 4 % paraformaldehyde and stored in the dark at 4 ˚C until analysis. Estimation of PBMC's proliferation was performed using a Beckman Coulter flow cytometer (FC500, Gladesville, NSW) and analysed using FlowJo software (Tree Star Inc., Oregon). Proliferation was expressed as the % of PBMC that had undergone two or more cell divisions.

Statistics

All statistical analyses were performed using Graph-Pad Prism version 5 (Graph Pad Software, LaJolla, CA, USA). Group A1, A3 and B1 data presented are the mean of 4, 4 and 8

koalas, respectively (± SE of the mean). Two-way ANOVA with Bonferroni's Post test with the *P* value set at < 0.05 was used to analyse the antibody titers in plasma and conjunctival swab samples. In vitro neutralization and PBMC proliferation studies were analysed using unpaired t-tests with the *P* value set at < 0.05.

## Results

### Evaluation of vaccine safety in healthy and diseased koalas

Of the 22 wild koalas involved in the trial (Table 1), sixteen received the vaccine plus adjuvant and six received the placebo (adjuvant alone). Ten animals (Group A) were not clinically affected by chlamydial disease (eight immunized, two placebo) while the remaining 12 koalas (Group B; eight immunized, four placebo) had been treated for clinical signs of chlamydiosis prior to inclusion in the trial. None of the koalas showed any adverse signs immediately after immunization or when observed for the following 48 hours. Furthermore, no koalas showed evidence of swelling or abscess formation at the vaccination site at subsequent monthly veterinary examinations up to 140 days (20 weeks) when the study was terminated.

### Evaluation of pathology in vaccinated, diseased and healthy koalas

Table 2 shows the clinical disease score for each vaccinated animal (vaccine versus placebo groups) following vaccination. Nine of the 12 Group B animals did not show any worsening of their clinical disease score when observed for up to 140 days following vaccination. Three koalas, all of which had cystitis, demonstrated progressive worsening of sonographic findings, specifically, increasing bladder wall thickening and persistent haematuria on at least one evaluation. Two of these received the placebo (three dose regime) while one received the vaccine.

### Vaccination elicits MOMP-specific IgG in healthy animals and boosts pre-existing immunity in diseased animals

Figure 1 demonstrates the enhancement of MOMP G IgG titers in healthy and diseased animals, following vaccination. Groups A1 (healthy animals, vaccine x3) and B1 (disease positive, vaccine x3) had developed strong plasma MOMP G IgG antibodies (A and B). Group

B1 animals had clinical signs of chlamydial disease on entry into the trial and not surprisingly therefore had pre-existing low anti-*Chlamydia* antibody levels on day 0 at both systemic and mucosal sites. In plasma, similar kinetics were observed in both the groups against MOMP G i.e. both the groups reached an end point titre (EPT) of $1 \times 10^5$ by day 84, which started to plateau beyond 20 weeks. At the ocular site (Fig. 1C and D), group A1 animals had minimal antibodies on day 0 and reached an EPT of $3 \times 10^2$ by day 140. However, B1 animals started with $1.6 \times 10^2$ EPT and reached a peak of $5 \times 10^2$ EPT by day 140. In both groups, vaccination induced a significant increase in MOMP IgG in plasma and secretions that was sustained out to day 140.

Figure 2 shows the enhancement of NrdB IgG titers in healthy and diseased animals. Although both the vaccine groups had stronger enhancement of NrdB antibodies in plasma from day 0, interestingly group A1 animals had only reached an EPT of $3 \times 10^4$, whereas B1 animals had reached its peak of $8 \times 10^4$ EPT by day 112 and dropped down later (Fig. 2A and B). At ocular sites, group A1 reached its peak of $3 \times 10^2$ EPT by day 112, whereas group B1 animals showed significant titers only from day 84 and reached a similar EPT of A1 by day 140 (Fig. 2C and D). Both MOMP G and NrdB antibodies for the two groups at both sites were statistically significantly higher by the end of the trial (20 weeks) with a *P* value set at 0.05.

Specificity of the antibody response following vaccination

Figure 3 shows immunoblot analysis of the immune-reactivity of plasma from vaccinated animals (all vaccinated using genotype G MOMP protein) against recombinant MOMP proteins of *C. pecorum* genotypes A, F, G and H. Animals given the placebo (groups A2 and B2) did not develop any new antibody response that was not present on day 0 of the trial, as expected. Group A1 animals (disease-free, vaccinated with genotype G MOMP) had developed antibodies by day 140 that recognised all four MOMP types. Group B1 animals (disease-positive) were all infected with genotype F *C. pecorum*, (data not shown) except one animal, which had a genotype G infection, prior to the start of the trial. Following immunization, these animals also developed antibodies that recognised all four MOMPs (A, F, G and H). Given that the recombinant MOMP G used for vaccination was a His-tagged protein and the genotype A, F and H MOMPs used in the Western blot were also His-tagged

proteins, a potential explanation for these results could be that the immunized animals produced a strong antibody response against the His tag common to all MOMPs and that the immunoassay is detecting reactivity against this tag rather than the MOMP itself. To rule out this possibility, we blotted day 140 plasma from animals in Group B1 against a *Legionella* TSP His-tagged protein (provided by Ms.Amba Lawrence/Dr.Willa Huston). As can be seen in Figure 3, plasma from MOMP G-immunized koalas had no reactivity against this non-chlamydial his-tagged protein.

Development of plasma neutralizing antibodies following vaccination

The percentage of in vitro neutralizing antibodies against *C. pecorum* genotype G was determined in the plasma samples (1/10 dilution) collected at day 0 and day 140. Plasma collected from each individual animal on day 0 of the vaccine trial was used as controls. All samples on days 0 and 140 were normalised to no plasma (infection only) controls. As shown in Figure 4, plasma from groups B1, A1 and A3, all immunized with MOMP G plus NrdB, had a significantly enhanced percentage of in vitro neutralizing antibodies compared to the relevant placebo control plasma (groups A2 and B2). Neutralizing activity against the immunising genotype G was significantly higher in animals that were disease free (A1) compared to animals that had a previous *C. pecorum* infection (B1, $p<0.05$).

Development of lymphocyte proliferation responses following vaccination

To assess the cell-mediated immune response following immunization, PBMC were collected on day 140 and labelled with CFSE. Proliferation was determined by dye-dilution assay following in vitro stimulation with MOMP G and NrdB as shown in Figure 5. Around 20 % of PBMC's in immunized healthy (Group A1) and 15 % in immunized diseased (Group B1) underwent two or more rounds of division following in vitro antigen stimulation. Cell proliferation (3 %) was also evident in the placebo control groups (A2 and B2). The results for both vaccine groups were statistically significant ($p < 0.05$) compared to their respective placebo groups.

Comparison of two versus three immunizations for the development of anti-*Chlamydia* immune responses

Because immunization of wild koalas will likely involve capture and release, which are both costly and traumatic for the animals, we compared immune responses in animals immunized three times on days 0, 28 and 56 with animals immunized twice on days 0 and 56. As can be seen in Figure 6, MOMP-specific antibody in plasma was higher in animals immunized three times at day 84 compared to those immunized twice, however, similar EPTs were seen in both groups at day 140 when the experiment was terminated. NrdB-specific antibody EPTs in plasma were significantly lower than MOMP-specific EPTs, however, at day 140 there was no difference in the EPT between animals immunized two and three times. Similar EPTs against both antigens were also seen in ocular swab secretions in animals immunized two and three times. Importantly, when plasma from both groups of animals were assayed for in vitro neutralizing activity against genotype G *C. pecorum*, there was no significant difference in activity between animals immunized with the different schedules. The percent of PBMCs induced to proliferate following in vitro stimulation with MOMP plus NrdB was also equivalent in animals immunized either two or three times.

## Discussion

*Chlamydia* continues to be major threat to the long-term survival of the koala. Recent studies suggest that addressing the issue of chlamydial disease will have a significant effect on populations that are already in decline [21-27]. Antibiotics administered by oral and subcutaneous routes have proven ineffective for the clearance of chronic chlamydial infections [28]. Furthermore, antibiotic treatment can adversely affect the unique microbial flora essential for digestion of eucalypts [29, 30]. Therefore, developing a vaccine appears to be the best approach for controlling *C. pecorum* infections in koalas.

Our previous vaccine study [15] was designed to test three adjuvants for safety and demonstrate both humoral and cell mediated immune responses against *C. muridarum* MOMP, CT512 and *C. trachomatis* serovar D NrdB antigens in healthy koalas. Although this work demonstrated strong humoral and cell mediated immune responses, it did not demonstrate (i) humoral immune responses to host specific *C. pecorum* antigens, (ii) neutralizing antibodies to host specific *C. pecorum* infections, or (iii) immune responses in wild koalas previously infected with *Chlamydia*. The current study was designed to evaluate the ability of a vaccine formulated with the koala *C. pecorum* genotype G MOMP, NrdB and the adjuvant ISC, to induce significant systemic and mucosal immune responses in wild koalas, both healthy koalas as well as koalas infected with *C. pecorum*. To our knowledge, this is the first report to assess a *Chlamydia* subunit vaccine in an animal species adversely affected by naturally occurring ocular and genital tract chlamydial infections.

Previous vaccine trials in mouse or guinea pig models have showed worsening of pathology due to cross reacting antigens such as the Hsp60, resulting in immunopathogenic consequences at ocular or female genital tract sites [31-33]. However, studies in mice and guinea pigs, using MOMP, have been shown to elicit partial protection [34-36]. In the current study, although diseased animals were treated with antibiotics well ahead of trial's commencement, they still had active *C. pecorum* ocular and urogenital tract infections. Detailed clinical observations were made during the trial period and none of the 22 animals were observed to have developed any adverse pathology due to the vaccine. One animal in the diseased placebo group was observed to have developed an increased bladder thickening, which was attributed to its current chlamydial infection (Table 2). Four animals

in group B1 had improved (decrease in disease score) gross clinical disease pathology over the study's time course, whereas no worsening (no change in disease scores) of the pathology was observed for the remaining animals in the group.

In this study, we were able to demonstrate that koalas already showing clinical signs of chlamydiosis had low level, pre-existing immune responses to *C. pecorum* MOMP and NrdB. This pre-existing response, however, did not prevent the development of strong vaccine-induced responses against heterologous MOMP from other *C. pecorum* genotypes nor the highly conserved antigen NrdB. Animals in groups B1 and B2 started with background IgG titers against both MOMP (Fig. 1) and NrdB (Fig. 2) in both plasma and ocular secretions, due to their previous infections. Pre-existing IgG levels against MOMP were higher than IgG anti-NrdB levels. Following subcutaneous immunization, both healthy and diseased koalas developed equally high levels of plasma anti-MOMP IgG antibodies (Fig. 1A and 1B) during the 140-day trial. However, MOMP-specific IgG titres in ocular swabs were higher in diseased animals than the healthy group at day 140 when the trial was terminated (Fig. 1C versus figure 1D) although this was not statistically significant. Following subcutaneous immunization, NrdB-specific IgG levels in plasma from diseased animals were significantly higher than in healthy animals (Fig. 2A versus 2B) whereas IgG anti-NrdB titres in ocular swabs were similar in both groups (Fig. 2C versus 2D). The vaccine-induced responses in both groups against MOMP were higher than that against NrdB, consistent with the highly immunogenic nature of MOMP. Importantly, the subcutaneous immunization route used was able to elicit both systemic (plasma) and mucosal (ocular) IgG responses. This data confirms the ability of naturally infected and diseased animals to elicit specific immune responses following immunization with our *C. pecorum*-specific vaccine candidate antigens. Importantly, it also shows that our recombinant vaccine antigens can stimulate the immune response of naturally infected animals, confirming that the presentation of our antigens is adequate for stimulation of responses relevant to whole C*. pecorum*.

Although we have shown that immunization elicits plasma IgG we have further determined if this antibody was able to inhibit chlamydial infection *in vitro*. To rule out any effect of pre-existing immunity due to natural infection (group B animals) or any effect of innate immune mediators, any neutralizing activity in pre-immune day 0 plasma was subtracted from the neutralizing activity of day 140 plasma. Plasma from immunized healthy animals resulted in

40% neutralization of *C. pecorum* G infectivity *in vitro* (Fig. 4A) whilst day 140 plasma from immunized, diseased animals was able to inhibit infection by 20% (Fig. 4B, $p<0.05$). This may be attributed to the disease status of the animals (i.e. the pre immune plasma from all the diseased animals had already low levels of neutralizing antibodies when compared to healthy animals. Hence, this is the first time a multi-subunit vaccine has shown the ability of to neutralize koala *C. pecorum* infections *in vitro*.

A major and perhaps promising aspect of our results was the observation of cross-reactivity of MOMP antibody responses from vaccinated healthy and diseased koala cohorts to different MOMP proteins (Fig. 3). Although there are major differences (up to 50 amino acid differences) between major *C. pecorum* amino types, current data suggests that immunization with MOMP genotype G can produce cross-reacting antibodies against several of the common genotypes affecting koalas. These findings were found to be consistent with studies in non-human primate models immunized with *C. trachomatis* nMOMP [37-38]. Bioinformatic analysis of predicted B cell epitopes in MOMP from the four genotypes of *C. pecorum* identified two major epitopes (more than 10 amino acids each) in the conserved regions of these MOMPs, which we predict may be responsible for producing cross-reacting antibodies (data not shown). The observation of cross-reactivity with a single MOMP protein will be important in our planning for vaccination of koala populations, infected with multiple genotypes of *C. pecorum* [17-18].

Protection against chlamydial infection in other animal models has been shown to be dependent on the induction of cell-mediated immunity, particularly interferon gamma - secreting CD4 T cells. In both groups of immunized koalas we were able to show, using the CFSE dye dilution assay that between 10 and 20% of cells within a lymphocyte gate underwent two or more rounds of cell division following in vitro stimulation with MOMP and NrdB (Fig. 5). Similar to the neutralization data (Fig. 4), the levels of lymphocyte proliferation were slightly lower in immunized, diseased animals, although this difference was not significant. This is likely due to all but one of these animals having been infected with genotype F whereas genotype G MOMP was used in the vaccine. We were unable to determine the cytokine profile of proliferating cells, nor their phenotype (CD4 or CD8) due to a lack of reagents available for the koala.

Use of an injectable vaccine in wild koala populations would require capture and release of animals, which is both traumatic for the animals, expensive and requires skilled animal handlers. For this reason, if fewer booster immunizations can be used to elicit protection this would represent a major advantage in a wildlife setting. We therefore compared immune responses after two and three immunizations. At the end of the 140 day trial, similar levels of IgG against both MOMP and NrdB were seen in plasma and ocular swabs using both regimes (Fig. 6), although, as expected, titers increased more rapidly in the three vaccination group. Importantly, levels of plasma neutralizing antibody activity and lymphocyte proliferation were also equivalent in the two and three-shot vaccine groups, suggesting that a single booster immunization was able to elicit similar immune responses as two booster shots.

In summary, we have shown that a multi-subunit chlamydial vaccine can be safely administered to both healthy koalas as well as koalas that have a previous or current chlamydial infection. Importantly, vaccination did not enhance pre-existing pathology and similar levels of systemic (plasma) and mucosal (ocular) antibodies and lymphocyte proliferation were achieved in both healthy and diseased koalas following vaccination. Neutralizing antibody in plasma of healthy animals was significantly higher than that in immunized/diseased animals. Importantly, plasma from both groups was able to react against MOMP from genotypes other than the immunising genotype suggesting that induction of immunity against multiple genotypes may be possible. Whilst there are obvious logistical problems associated with vaccination of wild koalas that need to be overcome, our results provide strong evidence that development of a chlamydial vaccine to protect this iconic marsupial is feasible.

Acknowledgements

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| Group | Immunization | Number of animals | Clinical signs of disease at start of trial | Koala Identifiers |
|---|---|---|---|---|
| A1 | Vaccine (3 Shot) | 4 | No | Whistler/Cedar/Lilly/Alfreda |
| A2 | Placebo (3 Shot) | 2 | No | AriBruce/Captain |
| A3 | Vaccine (2 Shot) | 4 | No | Sam/Satchmo/Ben/Mia |
| B1 | Vaccine (3 Shot) | 8 | Yes | Romeo/Maggie/Nixon/FelixPitt/Mitchy/Popeye/Wylie/Meryl |
| B2 | Placebo (3 Shot) | 4 | Yes | Katey/Juliette/Kathy/Thompson |

**Table 1 –** List of all koalas in the vaccine trial. Group A includes healthy, qPCR and serologically negative animals, further divided into A1 (three shot vaccine), A2 (placebo) and A3 (two shot vaccine). Group B includes diseased, qPCR and serologically positive animals, further divided into B1 (vaccine) and B2 (placebo).

| Animal Identifier | Complication/Site of Infection | Day 0 | Day 28 | Day 56 | Day 84 | Day 112 | Day 140 | Change in disease score at the end of the trial 20 weeks (+/-) | Average change |
|---|---|---|---|---|---|---|---|---|---|
| Group B1(Vaccine) | | | | | | | | | |
| Romeo | Conjunctivitis/Right Eye | 3 | 3 | 2 | 2 | 2 | 2 | -1 | - 0.74 |
| Nixon | Conjunctivitis/Left Eye | 2 | 2 | 2 | 0 | 0 | 0 | -2 | |
| Popeye | Conjunctivitis/Left Eye | 3 | 3 | 3 | 3 | 3 | 2 | -1 | |
| Felix Pitt | Conjunctivitis/Both Eyes | 2 | 2 | 2 | 2 | 2 | 2 | 0 | |
| Maggie | Cystitis/Urogenital tract | 2 | 2 | 2 | 0 | 0 | 0 | -2 | |
| Wylie | Cystitis/Urogenital tract | 3 | 3 | 3 | 3 | 3 | 3 | 0 | |
| Meryl | Cystitis/ Urogenital tract | 2 | 2 | 2 | 2 | 2 | 2 | 0 | |
| Mitchy* | Cystitis/ Urogenital tract | 2 | 2 | 3 | 2 | 2 | 2 | 0 | |
| Group B2 (Placebo) | | | | | | | | | |
| Katey* | Cystitis/ Urogenital tract | 3 | 3 | 2 | 2 | 3 | 3 | 0 | 0.25 |
| Kathy* | Cystitis/ Urogenital tract | 2 | 3 | 3 | 3 | 3 | 3 | +1 | |
| Juliette | Cystitis/ Urogenital tract | 2 | 2 | 2 | 2 | 2 | 2 | 0 | |
| Thompson | Conjunctivitis/Left Eye | 2 | 2 | 2 | 2 | 2 | 2 | 0 | |

**Table 2 –** Clinical disease score for all the group B (diseased) animals till 20 weeks. (* Increase in pathology during the trial)

**FIGURE LEGENDS:**

**Fig. 1 –** MOMP G antibody titers (IgG) in plasma and ocular swabs following vaccination in groups A1 (healthy animals) **[A and C]** and B1 (diseased animals) **[B and D]**. *n* = 4 animals in group A1 and 8 animals in group B1 at each time point. Two-way ANOVA was performed with Bonferroni's post tests. **P* 0.01 – 0.05, ** *P* 0.001 – 0.01, *** *P* < 0.001.

**Fig. 2 –** NrdB antibody titers (IgG) in plasma and ocular swabs following vaccination in groups A1 (healthy animals) **[A and C]** and B1 (diseased animals) **[B and D]**. *n* = 4 animals in group A1 and 8 animals in group B1 at each time point. Two-way ANOVA was performed with Bonferroni's post tests. **P* 0.01 – 0.05, ** *P* 0.001 – 0.01, *** *P* < 0.001.

**Fig. 3 –** Immunoblot analysis of the specificity of post immunized plasma to various rec MOMP amino types from animals belonging to various groups A1+A3, A2, B1 and B2 (reference blots shown); and false positive check with an anti-his blot against non chlamydial his-tagged protein.

**Fig. 4 –** Percentage of vaccine induced in vitro neutralization against *C. pecorum* genotype G infection in the plasma at 20 weeks post first immunization. Plasma on day 0 was used as a pre-immunized control. **A.** Healthy animal's vaccine groups (A1) vs. Placebo group (A2). **B.** Diseased animal's vaccine group (B1) vs. Placebo group (B2). Unpaired *t*- tests were performed to determine the significance of differences between the groups at 20 weeks post vaccination. **P* 0.01 – 0.05, ** *P* 0.001 – 0.01, *** *P* < 0.001.

**Fig. 5 -** CFSE labelled lymphocyte proliferation assay (CLPA) using PBMC stimulated with respective antigens (8ug/well) in all the animals, expressed as percentage of proliferating cells. **A.** Healthy animal's vaccine groups (A1) vs. Placebo group (A2). **B.** Diseased animal's vaccine group (B1) vs. Placebo group (B2). Unpaired *t*- tests were performed to determine the significance of differences between the groups at 20 weeks post vaccination. **P* 0.01 – 0.05, ** *P* 0.001 – 0.01, *** *P* < 0.001.

**Fig. 6 –** Antibody titers (IgG) in plasma and ocular swabs following vaccination in groups A1 vs. A3 against recombinant MOMP G (A and C) and recombinant NrdB (B and D); in vitro neutralization percentages against *C. pecorum* G infection and % of proliferating cells to vaccine in groups A1 vs. A3. *n* = 4 animals in each group at each time point (E and F). Two-way ANOVA was performed with Bonferroni's post tests on combined data at day 0 and day 140. Unpaired *t*- tests were performed to determine the significance of differences between the groups at 20 weeks post vaccination. **P* 0.01 – 0.05, ** *P* 0.001 – 0.01, *** *P* < 0.001.

**Fig. 1**

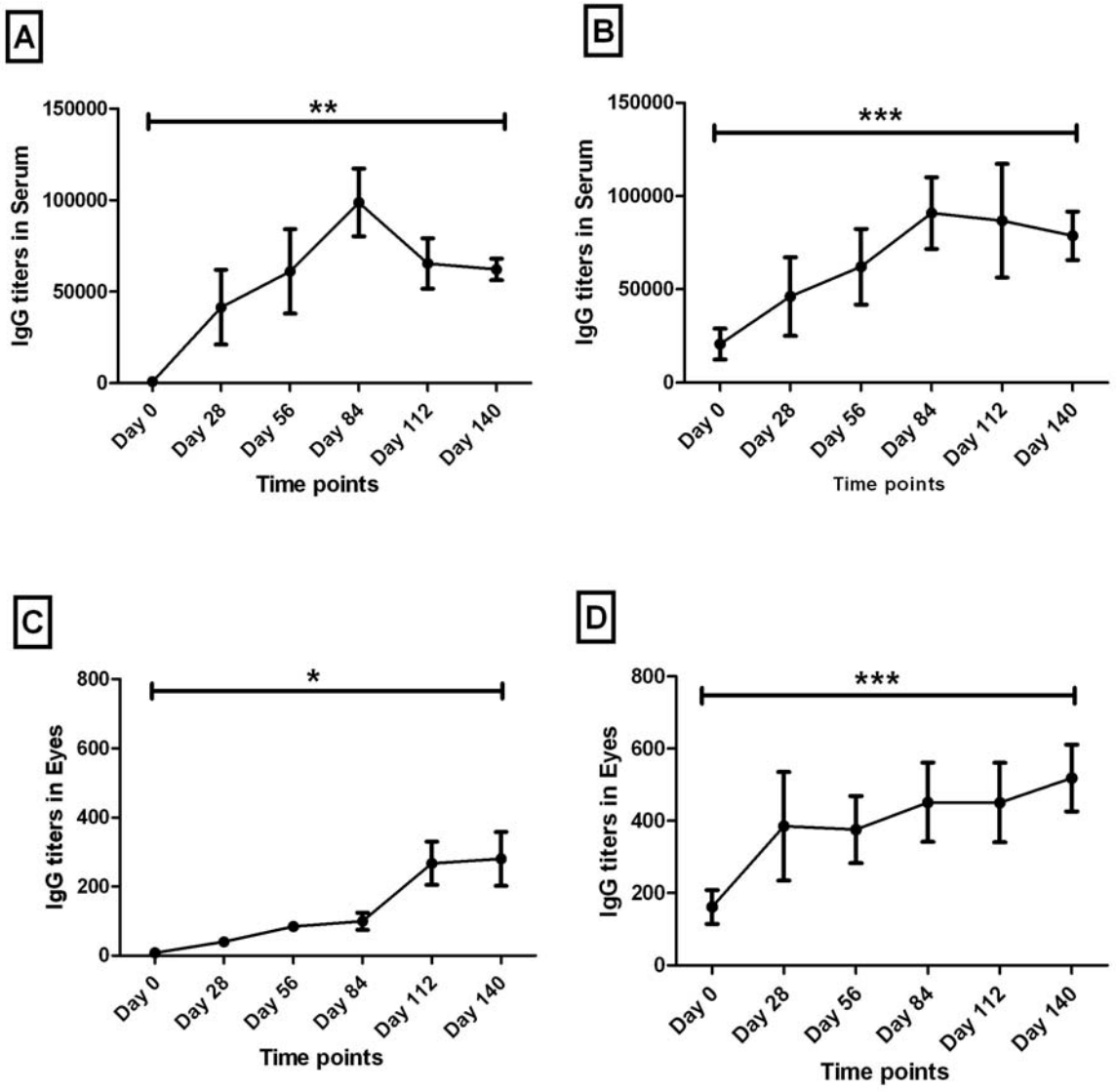
A
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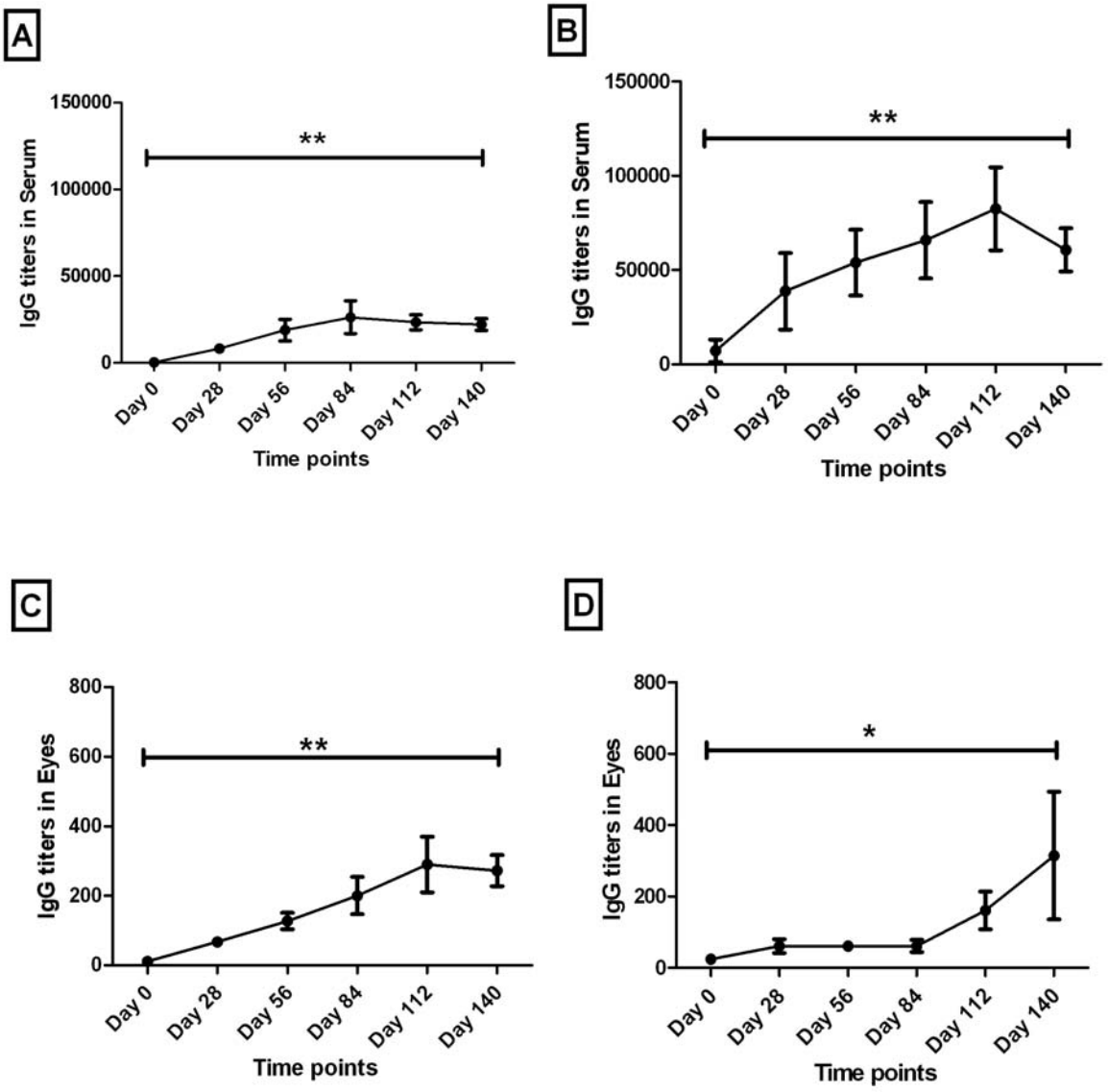
**Fig. 2**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Time points

**Fig. 3**

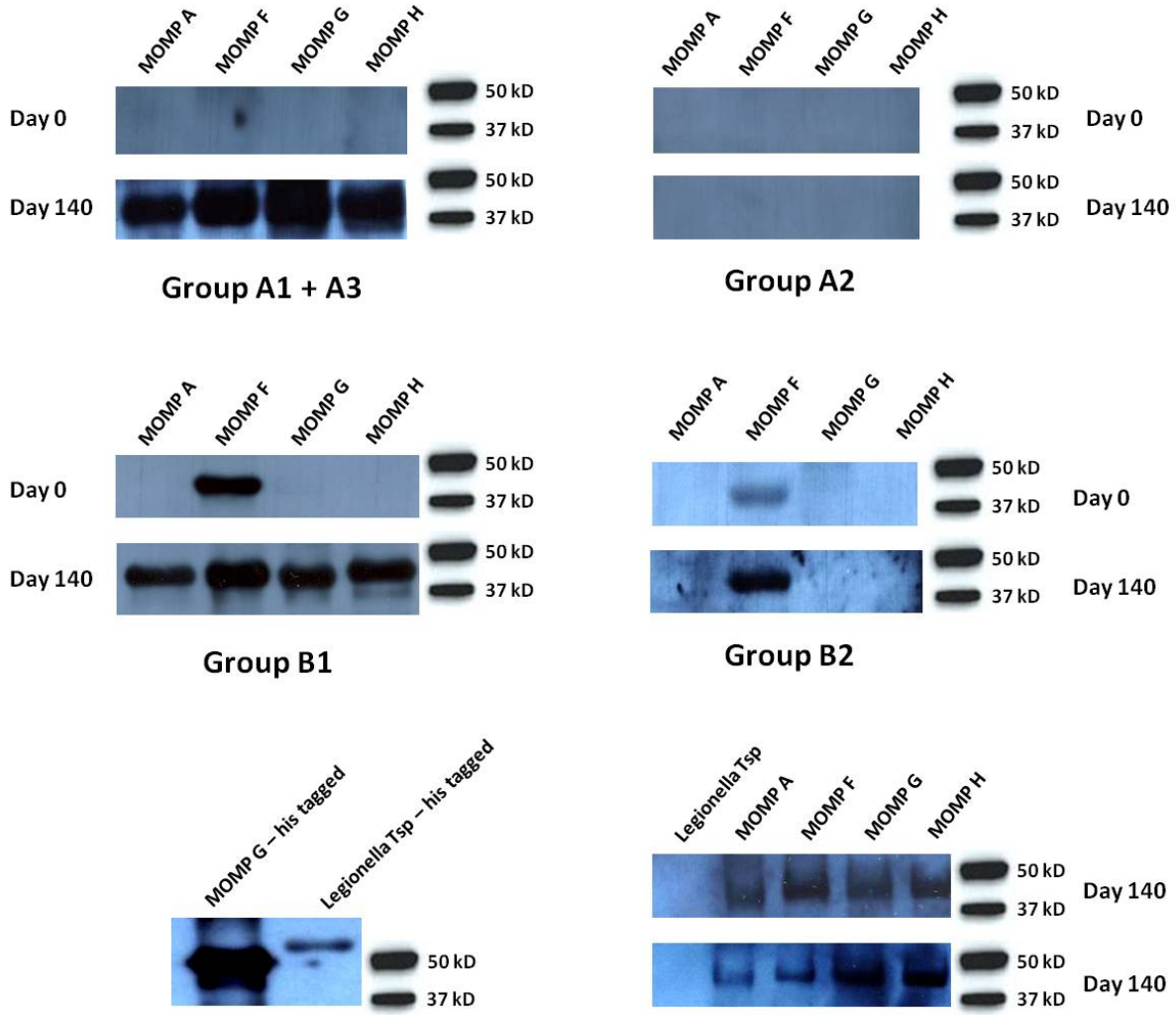
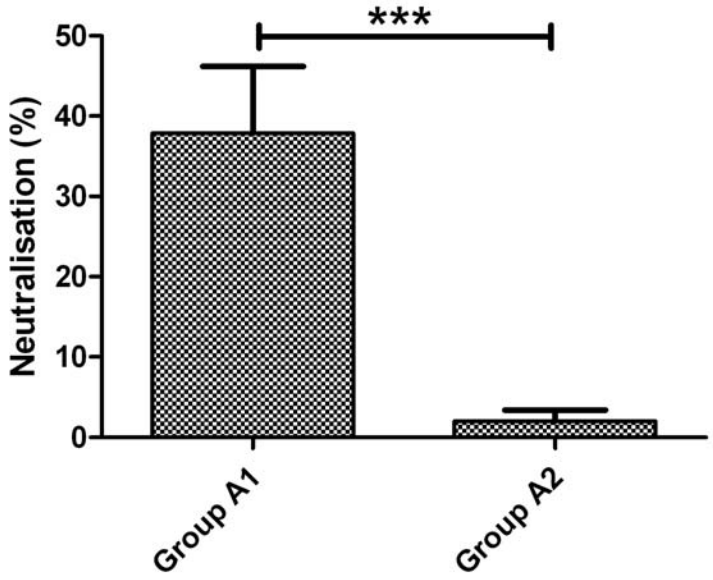

MOMP A
MOMP F
MOMP G
MOMP H
Day 0
Day 140
50 kD
37 kD
Group A1 + A3
Group A2
Group B1
Group B2
MOMP G – his tagged
Legionella Tsp – his tagged
Legionella Tsp

Fig. 4

A


***
50
40
30
20
10
0
Neutralisation (%)
Group A1
Group A2

B

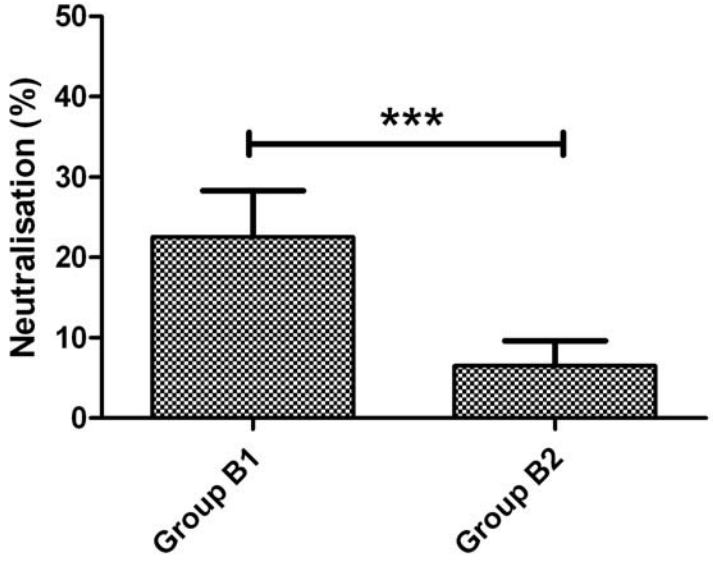
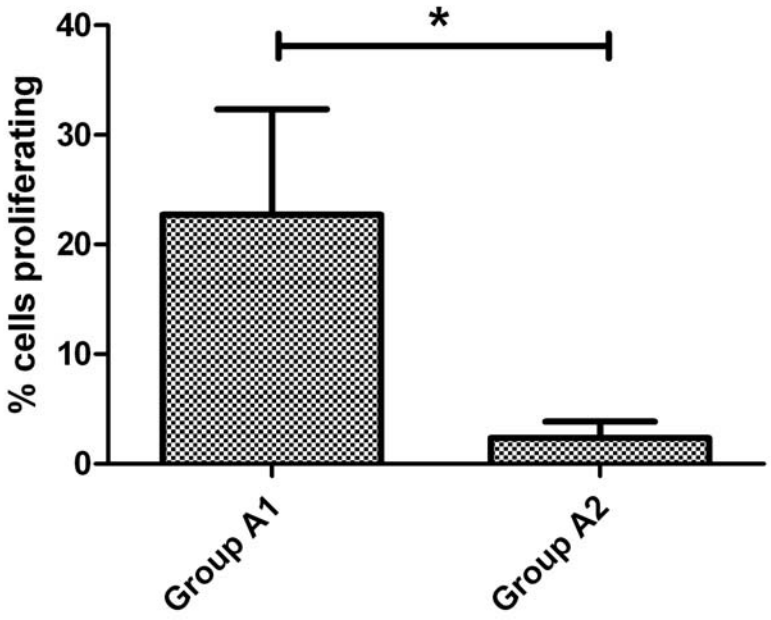

***
50
40
30
20
10
0
Neutralisation (%)
Group B1
Group B2

**Fig. 5**


*
40
30
20
10
0
% cells proliferating
Group A1
Group A2

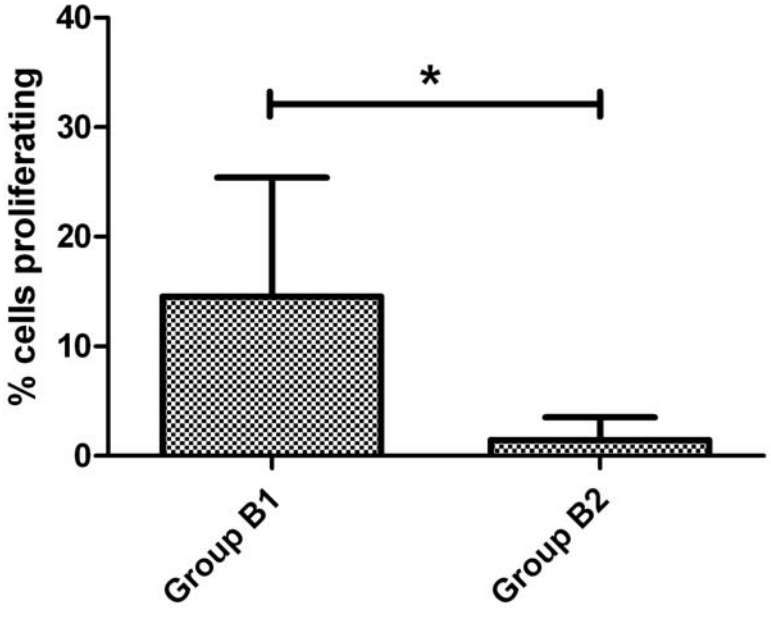
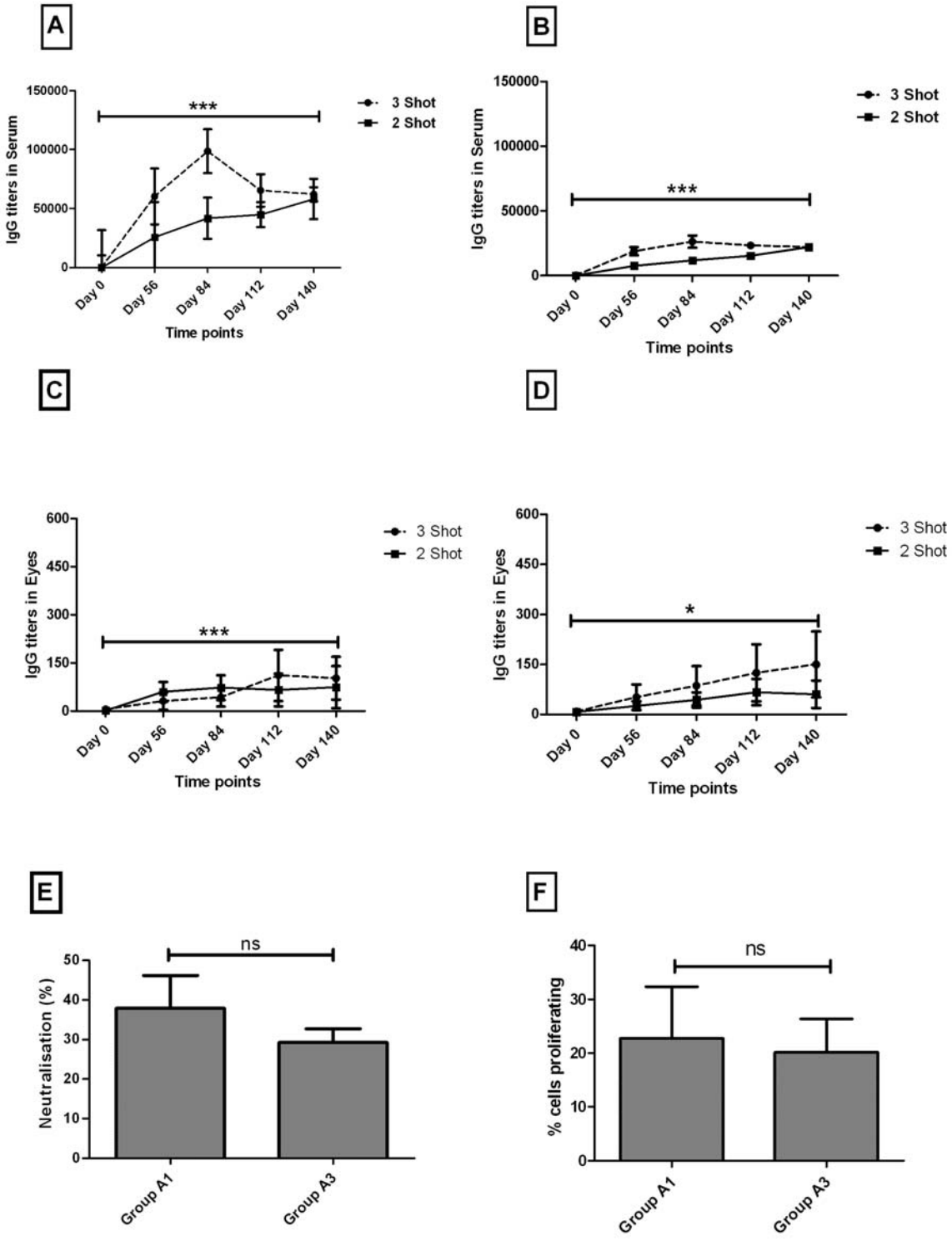

*
40
30
20
10
0
% cells proliferating
Group B1
Group B2

**Fig. 6**


A
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IgG titers in Serum
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3 Shot
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Time points
E
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40
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20
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0
Neutralisation (%)
ns
Group A1
Group A3
F
40
30
20
10
0
% cells proliferating
ns
Group A1
Group A3

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