# Insights from a different vantage point: New modelling of vaccination and epidemiology of *Chlamydia* infections


by

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## Thesis Abstract

*Chlamydia* are intracellular pathogens that infect a broad range of host species including humans and koalas. Depending on the bacterial strain, chlamydial infections can lead to severe reproductive or ocular disease, potentially resulting in infertility or blindness. Many infections are subclinical and may persist, complicating control strategies for this pathogen. Vaccination is a potential control strategy that could result in protecting vaccinated individuals against *Chlamydia*-related disease. The purpose of this thesis was to evaluate efficacy of candidate *Chlamydia* vaccines using novel biostatistical approaches to determine the effects of vaccination on measures of success, including changes in chlamydial load, host immune cytokine expression or anti-*Chlamydia* antibody production, and chlamydial disease. Vaccination success can be measured for its protective (against pathogen infection or disease from non-disease hosts) or therapeutic effect (reducing pathogen shedding or disease from diseased hosts). An additional research chapter using similar methodologies and focussing on *Chlamydia*, but departing from the vaccine focus, evaluated epidemiological factors likely to affect repeat chlamydial infection in women in Australia.

A chlamydial vaccine for humans does not yet exist but would be an ideal management strategy for controlling chlamydial infections. The number of vaccine-development studies published in recent years has made it difficult to determine trends and an objective review of the literature is important to identify the most promising vaccine candidate against chlamydial infection. In Chapter Two I performed a meta-analysis on systematically selected studies that aimed to develop a chlamydial vaccine either against *C. trachomatis* or another chlamydial species. Over 4,400 standardized effect sizes were calculated between control and chlamydial vaccination groups. Mice have most often been used in chlamydial vaccine

research (78%) and most vaccines against *Chlamydia* reduced chlamydial load and increased host immune parameter markers, including the antibodies IgA and IgG1, and the cytokine, IFNγ. Mice are frequently used in vaccine research due to the known inbred pedigree of each mouse, availability of the murine immunological toolkit, and a smaller demand of veterinary resources compared to non-mouse models. There are, however, limitations to mouse models of chlamydial infection that are used in experiments to develop a chlamydial vaccine for humans, most notably the unrealistically controlled conditions in laboratory settings.

Koalas are infected with *C. pecorum* in the wild and these infections have parallels with human *C. trachomatis* infections. In Chapter Three I investigated vaccine-immune-chlamydial load-disease relationships from previously collected data from MOMP (major outer membrane protein) vaccine trials in free-ranging koalas. Using structural equation modelling I created *a priori* hypotheses about perceived direct and indirect interactions from koalas vaccinated six months prior. I found MOMP vaccination had a strong effect on increasing interleukin 17 (IL17) mRNA expression, and that urogenital chlamydial load was positively associated with disease and negatively associated with IL17. Despite multiple potential sources of variation, owing to the koalas being free-ranging, these analyses helped illuminate a link between MOMP vaccination, urogenital chlamydial load and the cytokine IL17, enhancing previous investigations.

In Chapter Four I investigated individual variation in the immune response of koalas to MOMP vaccination, with a focus on immunoglobulin G (IgG) antibodies. I undertook this investigation in recognition that an ideal property of vaccines in development (from a veterinary and medical practitioner perspectives) is for them to elicit predictable immune

responses with minimal variation among individuals. However, many studies instead focus on average group effects (cohort effects). Using mixed effects models and methods adapted from the behaviour literature I examined previously collected IgG abundance data from koalas spanning three vaccine studies. I found significant heterogeneity in the individual variation of koala IgG levels in response to vaccination. Individual variation was minimised in vaccine trials undertaken on captive koalas measured over more timepoints after vaccination. This particular investigation presents strong evidence that chlamydial vaccine studies should consider examining both the average cohort effects and the individual variability in vaccine development trials.

Finally (Chapter Five), I undertook an epidemiological investigation of chlamydial reinfection risk in humans using similar structural equation modelling approaches to those used in Chapter three. Most genital chlamydial infections in humans can be treated with antibiotics, yet repeat infections of treated individuals in some populations remains significant (~20%). Multiple direct and indirect factors are associated with repeat infections and these associations are often complex and not well understood. I utilised data from the Australian Chlamydia Treatment Study, from which 239 women were recruited and 33 (13.8%) repeat infections were documented. My models confirmed that repeat chlamydial infections were predicted directly and positively by inconsistent condom usage. Importantly, I found repeat chlamydial infections were indirectly associated with participant age, use of anal sex, sexual network size, and vaginal sex frequency. These indirect factors highlight important factors for healthcare providers to consider for controlling repeat chlamydial infections.

My PhD research has advanced our understanding of the efficacy of candidate chlamydial vaccines and aspects of chlamydial epidemiology. The findings have contributed to: 1) the

identification of promising directions toward the development of a chlamydial vaccine, 2) the direct and indirect factors associated with chlamydial disease, 3) the individual variability among systemic antibody responses to vaccination, and 4) the direct and indirect factors associated with repeat genital *Chlamydia* infections. These findings have been achieved through the use of novel biostatistical approaches in the chlamydial research field, and through the application of these techniques to existing laboratory, wildlife and human studies. More broadly, the modelling approaches used in this thesis are also applicable to other fields of vaccinology and epidemiology.

# Chapter 1: General introduction

## 1.1 Impacts of sexually transmitted infections

Globally, sexually transmitted pathogens greatly impact humans, wildlife, and domesticated animals. From a public health perspective, these pathogens remain prevalent with varying rates that depend on a number of factors within a population (e.g., demography and socioeconomic status), despite the advancements of modern medicine. In 2012, there were approximately 417 million people infected with herpes simplex virus 2 (HSV-2), 291 million women infected with human papillomavirus (HPV), and 357 million new cases of four curable sexually transmitted pathogens (*Chlamydia trachomatis, Neisseria gonorrhoeae*, *Treponema pallidum*, and *Trichomonas vaginalis*; World Health Organization 2016). Infection with a sexually transmitted pathogen may result in pregnancy complications (Wynn et al. 2020), increased risk of some cancers or human immunodeficiency virus infection (Caini et al. 2014; Nusbaum et al. 2004), infertility (Anyalechi et al. 2019), and/or significant impacts to an individual's physical, psychological, or social wellbeing (Frost et al. 2007). From an agricultural perspective, sexually transmitted pathogens may lead to lower productivity, extended breeding and calving seasons, calf losses, and considerable costs for treatment and prevention of infections within animal stocks (Michi et al. 2016). From a wildlife ecology perspective, sexually transmitted infections may contribute to population losses in the wild, affecting animal population dynamics. Infected wildlife may be reservoir hosts to pathogens with zoonotic potential and are relevant to the One Health approach of improving public health (Jelocnik 2019). The consequences introduced here, highlight the importance of understanding sexually transmitted infections, particularly pathogens that are significant sources of disease.

### 1.2 Symptoms of *Chlamydia*-related disease

*Chlamydia* are significant pathogens to a broad range of hosts (Borel et al. 2018). In humans, the majority (~80%) of genital *Chlamydia* (*C.*) *trachomatis* infections remain subclinical and can persist for extended periods without signs or symptoms (Peipert 2003). Untreated genital infections that progress to disease can range in effect from varying levels of discomfort all the way to severe reproductive complications in both women and men. In women, acute symptoms typically include mucopurulent discharge and post coital bleeding. Over time, infections in women may develop into pelvic inflammatory disease (PID) and scarring of the fallopian tubes (salpingitis), which can lead to ectopic pregnancy and infertility (Peipert 2003). At the mild end of complications, men may experience symptoms including painful urination and urethral discharge. In men, long term complications can include impaired sperm development and infertility (Cunningham and Beagley 2008). Additionally, within the men who have sex with men community (and in some rare cases, women), rectal chlamydial infections have led to symptoms including anorectal discomfort, bleeding, mucopurulent anal discharge, and painful bowel movements from proctitis (Leeyaphan et al. 2016; Peuchant et al. 2011).

It is well known that *Chlamydia* can infect humans via sexual transmission, though not all human chlamydial infections are transmitted this way. Ocular *C. trachomatis* infections often occur at a younger age and may be transmitted via ocular secretions, fomites, or eye seeking flies (Emerson et al. 2004). Ocular infections can cause irritation of the eyes and may result in ocular conjunctivitis. Untreated and repeated infections may lead to scarring of the conjunctiva, known as trachoma, and is the leading cause of preventable blindness in the world (Mariotti et al. 2009).

The diseases caused by *C. trachomatis* in humans are similar to the diseases caused by other chlamydial strains in non-human animal hosts. Koalas (*Phascolarctos cinereus*) infected with ocular *C. pecorum*, for example, can suffer from ocular conjunctivitis and, in severe cases, inversion of the eyelid resulting in blindness (Quigley and Timms 2020; Wan et al. 2011). Urogenital *C. pecorum* infections can result in incontinence resulting from cystitis, scarring of the fallopian tubes, and ultimately infertility (Quigley and Timms 2020; Wan et al. 2011). Unlike *C. trachomatis*, *C. pecorum* infections are much more likely to result in chlamydial disease in some animals (Robbins et al. 2019). For example, estimates of disease prevalence in wild koala populations range between 26% and 100% (Loader 2010; Polkinghorne et al. 2013; Quigley and Timms 2020). Lastly, some *Chlamydia*-related disease signs are more uniquely observed in domesticated animals. *C. pecorum* infections in sheep and cattle have been shown to result in cases of polyarthritis and encephalomyelitis (Walker et al. 2015; Walker et al. 2016). The diverse, and sometimes devastating, range of signs and symptoms generated by chlamydial infection and disease in a range of hosts makes this an important pathogen for study.

### 1.3 Host diversity of the *Chlamydiales*

All chlamydial species fall under the order *Chlamydiales*. This order contains the family *Chlamydiacea* (under which the major human and animal pathogen sit) and eight other recognized families of *Chlamydia*-like organisms (Burnard and Polkinghorne 2016). The family *Chlamydiaea* consists of the following eleven recognized species (with their natural hosts): *C. pneumoniae* (multiple eutherians including humans, marsupials, amphibians and reptiles), *C. pecorum* (eutherians and marsupials), *C. trachomatis* (humans), *C. muridarum* (mice), *C. suis* (eutherians, particularly pigs, and amphibians), *C. caviae* (eutherians,

particularly guinea pigs), *C. felis* (cats), *C. abortus* (eutherians, amphibians, birds), *C. psittaci* (eutherians including humans, amphibians, birds), *C. gallinacea* (birds), and *C. avium* (birds).

Members of the *Chlamydiales* appear to exist in hosts from every ecosystem around the world (Collingro et al. 2020), with Australian hosts particularly well studied (Jelocnik 2019). Within wild marsupials, a 2003 survey detected *Chlamydiacea* 16S rRNA (referred to in the study as the proposed reclassification of the family, *Chlamydiales*) in Greater Gliders (*Petauroides volans*), Mountain Brushtail Possums (*Trichosurus caninus*), Western Bar Bandicoots (*Perameles bouganville*), Greater Bilbys (*Macrotis lagotis*), and Gilberts' Potoroos (*Potorous gilbertii*) with some evidence of ocular disease in these animals (Bodetti et al. 2003). A 2017 study expanded upon this work and showed evidence of *Chlamydiacea* 16S rRNA positive samples from 10 different marsupial species from sites surveyed along the east coast of Australia, the Northern Territory, and Tasmania (Burnard et al. 2017). Interestingly, *Chlamydiacea* positive samples could be obtained from engorged ticks collected from Australian wildlife, though they seem unlikely to act as vectors of chlamydial organisms (Burnard et al. 2017). However, within the group of Australian marsupials, the most commonly studied is by far the koala. Koalas are a major focus of chlamydial disease ecology because of recent population declines in Queensland and New South Wales, Australia, resulting in their conservation status being listed as 'vulnerable' in these regions (Woinarski et al. 2015). Koalas are hosts to two different species, *C. pecorum* and *C. pneumoniae*, with *C. pecorum* infections being more common in the wild (Polkinghorne et al. 2013).

Beyond Australian marsupials, members of the family *Chlamydiacea* are pathogens for domesticated livestock world-wide, particularly *C. pecorum* in cows, sheep, and pigs, *C.*

*abortus* in cows, sheep, pigs, and horses, *C. psittaci* in birds, and *C. suis* in pigs (Everett 2000; Reinhold et al. 2011). Though hosts can be infected with multiple chlamydial species, usually a single species is dominant among hosts in high density populations (Li et al. 2016). For example, *C. pecorum* detection is so common among cattle (particularly in the gastrointestinal tracts) that it is possibly endemic (Li et al. 2016). Positive *Chlamydiaceae* samples have been detected from herds in countries around the world, including Australia (Bommana et al. 2017), Austria (Petit et al. 2008), Germany (Biesenkamp-Uhe et al. 2007), Italy (Cavirani et al. 2001), Sweden (Godin et al. 2008), Switzerland (Ruhl et al. 2009), Taiwan (Wang et al. 2001), and the Unites States (Jee et al. 2004). In some instances, these livestock infections may have been the result of spillover and an epizootic event. For example, in 2016, an epizootic *C. psittaci* strain was associated with multiple abortions among thoroughbred horses in New South Wales, Australia (Jelocnik et al. 2018). Molecular evidence suggested that this strain, 6BC, could have originated from native Australian psittacines (Jenkins et al. 2018). As molecular biologists continue to monitor both livestock and wildlife in the same area, it will be interesting to determine both the frequency of these events and factors associated with their occurrence.

This thesis will focus primarily on three chlamydial species, *C. trachomatis* (in humans and humanised mice), *C. muridarum* (in mice as a model for *C. trachomatis*) and *C. pecorum* (in koalas).

### 1.4 Biology of *Chlamydia*

*Chlamydia* are gram negative, intracellular bacteria with a biphasic lifecycle (Elwell et al. 2016). They exist outside of host cells as highly infectious, extracellular elementary bodies (EBs) ~0.2 to 0.3 µm with a reduced metabolic activity. Chlamydial elementary bodies are

encapsulated with a number of proteins, including the major outer membrane protein (MOMP) and the peripheral membrane protein (Pmp). MOMP is a 40-kDa dominant surface protein covering ~60% of the outer chlamydial membrane, made up of four variable domains (VDs) and five constant domains. The ompA gene coding for MOMP is highly polymorphic, making it a common marker to define different chlamydial genotypes (Kaltenboeck et al. 1993). *C. trachomatis* can be broadly described based on their site of infection (biovars) and further described by genotype (based on MOMP sequencing): ocular biovars, (genotypes A to C), genital tract biovars (genotypes D to K), and lymphogranuloma biovars (L1 to L3; Elwell et al. 2016). *C. pecorum*, though not defined by biovars, has 15 unique genotypes. Within *C. muridarum*, there exists one MOMP allele (Read et al. 2000), whereas *C. trachomatis* and *C. pecorum* have multiple MOMP alleles (Kaltenboeck et al. 1993). Structurally, MOMP is a trimer made of β- pleated sheets and functionally acts as a porin (Sun et al. 2007). Pmp is a ~100 to 150-kDa protein that functionally acts as an autotransporter adhesin and is associated with chlamydial virulence (Becker and Hegemann 2014). Chlamydial species can express several different Pmps, depending on the gene number, with *C. trachomatis* containing nine, *C. muridarum* containing nine, and *C. pecorum* containing 15 (Vasilevsky et al. 2014).

Chlamydial elementary bodies primarily target and enter mucosal epithelial cells at either the respiratory, lymphoid, genital, or ocular sites (Brunham and Rey-Ladino 2005). They use needle-like type three secretion systems (TTSS) to deliver virulence effectors into the host cytosol. Chlamydial EBs enter the host cell through a membrane-bound vesicle (i.e. an inclusion) outside of the endocytic pathway, effectively avoiding lysosomal degradation. Once inside the vesicle, the EB transitions into a larger reticulate body (RB), ~0.8 µm, with

increased metabolic activity. The RB, still attached to the inclusion, secretes inclusion proteins (Incs) that transverse the inclusion membrane and are responsible for communication with the host cell and nutrient acquisition. In addition to Incs, the RB also produces a number of factors including the chlamydial protease activity factor (CPAF). CPAF is a protease that degrades host transcription factors for major histocompatibility complex (MHC) gene activation (Shaw et al. 2002). The RB divides by binary fission and produces EBs within the inclusion. During periods of stress (e.g. host immune mediated reduction in nutrients), the RBs may enter a persistence form (Beatty et al. 1994). This persistence form (termed an 'aberrant body') is non-dividing and silent within the cell, effectively evading the stressor. Upon the return of more favourable conditions, the aberrant bodies reactivate into RBs. At the final stages of their lifecycle, RBs transition into EBs and exit the cell (along with any secreted factors) to infect other host cells.

**1.5 Chlamydial treatment**

Most non-complicated chlamydial infections in humans can be treated with antibiotics, either azithromycin (single 1g dose) or doxycycline (7 days of 100mg dose twice a day; Workowski and Berman 2010). Azithromycin is often prescribed to avoid compliance issues sometimes observed with the longer doxycycline treatment. Recent meta-analyses, however, show a lower efficacy with azithromycin compared to doxycycline treatment in clearing genital (Kong et al. 2014) and rectal (Kong et al. 2015) chlamydial infections. Treatment for repeat genital chlamydial infections after clearance is estimated to be high, with ~25% of patients in some populations returning for treatment within one year (Gaydos et al. 2008; Kampman et al. 2016; Rose et al. 2020). While *Chlamydia* may develop drug resistance *in vitro* (Suchland et al. 2009), the high physiological cost of obtaining macrolide

resistance has been shown to be associated with a reduction in fitness. Thus, antibiotic resistant chlamydial strains are unlikely to arise *in vivo* (Binet and Maurelli 2007). There are a number of factors known to be directly or indirectly associated with repeat chlamydial infections, such as risky sexual behaviour, persisting chlamydial strains, size and frequency a sexual network is accessed, autoinoculation from infections in the gastrointestinal tract (Craig et al. 2015; Hocking et al. 2013). Indeed, these highly complex relationships remain poorly understood.

In koalas, antibiotic treatment of chlamydial infections is possible, but complicated. Koalas harbor a unique gastrointestinal microflora required to digest eucalyptus. Antibiotic administration may alter this microflora, resulting in dysbiosis that can be fatal for koalas (Polkinghorne et al. 2013). One antibiotic, chloramphenicol, is commonly used to treat chlamydial infections (60 mg/kg for 14 to 28 days; Robbins et al. 2018). Dependency on this antibiotic has become problematic in recent years as commercial supplies of chloramphenicol are becoming unreliable and other antibiotics to treat koala chlamydial infections have produced mixed results (Quigley and Timms 2020).

**1.6 Chlamydial vaccine development**

Vaccines are a promising alternative to antibiotics for controlling both *C. trachomatis* (Brunham and Rey-Ladino 2005; de la Maza et al. 2017) and *C. pecorum* infections (Polkinghorne et al. 2013; Quigley and Timms 2020; Waugh and Timms 2020). At present, there are currently no vaccines for *C. trachomatis* or *C. pecorum* species commercially available. Vaccines are considered the most promising avenue for this pathogen because they can be both protective, by enhancing the host immune system to recognize the pathogen to prevent infection (Brunham and Zhang 1999; Buendia et al. 2009; Carey et al.

2011) or disease (Bulir et al. 2016) and, therapeutic, by reducing the severity of infection or disease in affected hosts (Biesenkamp-Uhe et al. 2007; Nyari et al. 2019).

*Chlamydia* vaccine formulations were traditionally composed of attenuated or inactivated pathogen cells (e.g., inactivated poliovirus vaccine). In the 1960s, two prototype *C. trachomatis* vaccines consisting of either inactivated or live chlamydial EBs, were tested in major human clinical trials in Saudi Arabia, The Gambia, India, and Ethiopia aimed at reducing trachoma (reviewed by Mabey et al. 2014). The results from these trials showed that the vaccines offered short-term protection (~6 months post vaccination) from trachoma, but no long-term protection (12-24 months post vaccination). After the completion of these trials, chlamydial vaccines were shifted to non-human pre-clinical research trials (Phillips et al. 2019).

Following the success of HPV and Hepatitis-B subunit vaccines (Markowitz et al. 2016), it was realized that a chlamydial subunit vaccine might have promise for eliciting host immune responses and protection without the risks of chlamydial disease from intact EBs (Frietze et al. 2018). Tested chlamydial subunit vaccines often consist of external chlamydial proteins as antigenic targets (e.g., MOMP, Pmp, CPAF) and can be delivered along with immune-stimulating adjuvants (Phillips et al. 2019). Mouse models were the most common hosts used to test novel *C. trachomatis* vaccines (including vaccines against *C. muridarum* in the mouse model; Lizárraga et al. 2019; de la Maza et al. 2021), due in large part to lower costs and the availability of the immunological reagents and inbred mouse lines that allowed researchers to control the genetic variation between infected hosts (Farris et al. 2011; Vasilevsky et al. 2014). Often, these trials reported a number of measures of success including changes in chlamydial load (i.e. abundance of chlamydial organisms) after a

challenge (Tifrea et al. 2020), measurements of the immune system (including cell signalling cytokines and anti-*Chlamydia* antibodies; Pal et al. 2017), and measurements of disease (Bulir et al. 2016). As the number of chlamydial vaccine trials continues to grow ((Lizárraga et al. 2019; Phillips et al. 2019), it becomes increasingly important to not only evaluate overall cohort vaccination success, but to also evaluate which vaccines consistently elicit protection against *Chlamydia* to guide future research towards a commercial vaccine.

Vaccines against *C. pecorum* have been trialled in domesticated animals (e.g., cows, sheep, and pigs; Desclozeaux et al. 2017b) and koalas as a promising measure of disease control (Waugh and Timms 2020). To date, 11 *C. pecorum* vaccine trials have been conducted using either captive or free-ranging koalas (recently reviewed by Phillips et al. 2019). Similar to *C. trachomatis* infections in humans, multiple *C. pecorum* MOMP genotypes have been reported from infected koalas in the wild (Kollipara et al. 2013; Marsh et al. 2011). Two large field vaccine trials ($\geq$60 koalas) were recently conducted by Khan et al. (2016) and Desclozeaux et al. (2017a). Waugh et al. (2016) tested a recombinant MOMP vaccine consisting of three genotypes common in southeast Queensland koala populations (genotypes A, F, and G) that was delivered along with an immune-stimulating complex adjuvant. The results from this trial showed that the vaccine could reduce chlamydial load and disease in vaccinated animals (i.e. a therapeutic effect) compared to non-vaccinated animals and that it increased the systemic immunoglobulin G (IgG) production (i.e. one measure of protection) in vaccinated animals by up to six months post vaccination. Desclozeaux et al. (2017a) tested both a recombinant MOMP vaccine (also with genotypes A, F, and G) and separately a recombinant Pmp (genotype G) vaccine that were both delivered alongside three adjuvants: IDR-1002 (an anti-inflammatory), PCEP (an

immunogenic protein carrier), and Poly I:C (promotes host cytokines). This trial showed that both single-dose vaccines increased the production of anti-*Chlamydia* IgG antibodies and the host cytokines interferon gamma (IFNγ) and interleukin 17 (IL17) in the majority of vaccinated animals. Interestingly, MOMP vaccinated animals had a significant reduction in chlamydial load at six months post vaccination, with Pmp vaccinated animals showing comparable infection loads to the non-vaccinated control animals. Following these large field trials, a smaller trial with captive healthy koalas by Nyari et al. (2018) showed that synthetic MOMP peptides could elicit similar systemic IgG responses up to six months post vaccination compared to full-length recombinant MOMP. The results from this study indicate that these vaccines elicited one measure of protection (systemic IgG), however, the animals in this trial were not infected or challenged with *Chlamydia* making it difficult to determine whether these vaccines are protective and/or therapeutic or not.

### 1.7 Analytical approaches used to measure vaccine success

Veterinarians and clinicians seek the most effective vaccines available based on the results from vaccine trials (Brunham and Rappuoli 2013; Genovese et al. 2018; Malagón et al. 2012; Osterholm et al. 2012). There exist a number of approaches that have been previously used to evaluate the outcome of a vaccine trial (Mehrotra 2006; Nauta 2010). In almost all vaccine trials, a cohort effect is usually calculated as either a mean or median (± error) of some measure of vaccine success (e.g., chlamydial load) for vaccinated and control group comparisons (e.g., Badamchi-Zadeh 2016). In most cases, univariate analyses are used to statistically compare unvaccinated to vaccinated individuals (e.g., Waugh et al. 2016). In trials where longitudinal data exists, comparisons are made between measurements collected at baseline and a timepoint after vaccination. Waugh et al. (2016) used non-

parametric Wilcoxin signed-rank tests to evaluate differences in koala IgG antibody titer measurements at baseline versus six months post vaccination. They also used chi-squared contingency tables to evaluate changes in *C. pecorum* load using ordinal categories to classify these changes. Desclozeaux et al. (2017a) used Wilcoxin ranked tests to evaluate IgG responses between baseline and six months post vaccination, and Fisher's exact test to evaluate changes in chlamydial disease status before and after vaccination. Nyari et al. (2018) used a one-way analysis of variance (ANOVA) with a post-hoc Tukey's multiple comparison test to determine if there was an effect (and at which timepoint) of vaccination on IgG or IgA production after vaccination. In all three studies, appropriate analyses were used to evaluate direct effects of vaccination on a single measurement of success.

While most vaccine and epidemiological studies evaluate direct effects, such as those mentioned above, it is also widely acknowledged that relationships are complex. For example, direct and indirect effects of chlamydial vaccination on multiple measures of vaccination success are generally not accounted for in univariate analyses. Furthermore, any changes to these measures of success at the individual level are lost when individual measures of success are averaged to obtain the cohort effect. For example, it generally remains unclear 1) how MOMP vaccination directly or indirectly affects the immune-chlamydial abundance-disease relationships and 2) how variable vaccinated individuals are to one another in vaccine trials. Additionally, and with particular regard to the complex field of *C. trachomatis* vaccine development, it can be difficult to objectively identify the most promising vaccine candidates.

The effect size, or the difference in magnitude between vaccinated and control animals for a given measure of vaccination success, can be estimated and compared across trials to

highlight promising directions amid a growing number of vaccine trials (method described by Hedges (1981). Though meta-analyses have previously been used to estimate the proportion of community acquired pneumonia caused by chlamydial infections (Hogerwerf et al. 2017) and the association between HPV and *C. trachomatis* risk in women (Naldini et al. 2019), prior to my Ph.D. there existed no meta-analysis that compares the effect sizes of all chlamydial vaccine trials.

**1.8 Thesis aims and approaches**

The purpose of this thesis is to evaluate *Chlamydia* vaccine efficacy using previously collected data and applying novel biostatistical approaches to determine the effects of vaccination on measures of success including changes in chlamydial load, host immune cytokine expression or anti-*Chlamydia* antibody production, and chlamydial disease. One additional research chapter, using similar methodologies but departing from the vaccine focus, will evaluate direct and indirect factors likely to affect chlamydial reinfection in women in Australia with a focus on sexual practices. My thesis includes the following four data-driven chapters:

Chapter 2: A meta-analysis of the literature to highlight the most effective chlamydial vaccine across different hosts and against different species of *Chlamydia* (data sourced from 165 published studies, see Supplementary Figures 2.1 and 2.2)

Chapter 3: A structural equation modelling approach to understand the direct and indirect relationships underpinning vaccination success in free-ranging koalas (data sourced from Waugh et al. 2016; Desclozeaux et al. 2017)

Chapter 4: A mixed effects modelling approach to evaluate individual variability of koala immune responses to chlamydial vaccinations (data sourced from Waugh et al. 2016; Desclozeaux et al. 2017; Nyari et al. 2018)

Chapter 5: A structural equation modelling approach to investigate the direct and indirect factors that contribute to persistent or re-infections of *Chlamydia trachomatis* following antibiotic treatment in women (unpublished data sourced from J. Hocking; see published study protocol, Hocking et al. 2013)

As a final sixth chapter, I synthesize the results from these four chapters to discuss the implications of the biostatistical modelling approaches used in each chapter, limitations, and directions for future chlamydial vaccine research.

## Chapter 2: Navigating to the most promising directions amid complex fields of vaccine development: a chlamydial case study

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**Author contributions:** All authors conceived the study design, DL performed the systematic review and meta-analysis, and all authors contributed to interpreting the results and drafting of the manuscript.

## 2.1 Abstract

**Introduction**: Vaccine development research is proliferating making it difficult to determine the most promising vaccine candidates. Exemplary of this problem is vaccine development against *Chlamydia*, a pathogen of global public health and financial importance.

**Methods**: We systematically extracted data from studies that included chlamydial load or host immune parameter measurements, estimating 4,453 standardized effect sizes between control and chlamydial immunization experimental groups.

**Results**: Chlamydial immunization studies most often used (78%) laboratory mouse models. Depending on chlamydial species, single recombinant protein, viral and bacterial vectors, dendritic transfer, and dead whole pathogen were most effective at reducing chlamydial load. Immunization driven decrease in chlamydial load was associated with increases in IFNg, IgA, IgG1 and IgG2a. Using data from individual studies, the magnitude of IgA and IgG2a increase was correlated with chlamydial load reduction. IFNg also showed this pattern for *C. trachomatis*, but not for *C. muridarum*. We also reveal the chlamydial vaccine development field to be highly biased toward studies showing these effects, limiting lessons learned from negative results.

**Conclusions**: Most murine immunizations against *Chlamydia* reduced chlamydial load and increased host immune parameters. The meta-analysis in this chapter is novel for vaccine development and is critical in identifying trends where large quantities of literature exist.

Keywords: effect size, immunization, meta-analysis, muridarum, systematic search, trachomatis

## 2.2 Introduction

Modern molecular and computational techniques have led to an explosion of quantitative results in vaccine development publications. Vaccinology studies often aim to answer the following critical questions: 1) which immunizations are most successful, 2) what are some of the emergent trends from successful immunization studies using different animal hosts as models for infection, and 3) what effects do successful immunizations have on the host immune system? In the face of expanding literature, objective assessments of these questions can become overshadowed by variation in methodology among studies. Due to the growing cost and regulation surrounding vaccine trials in humans, objective determinations of the most promising vaccine types are therefore more valuable than ever. Emblematic of the field of vaccine development for many pathogens of public health importance (e.g. *Treponema*, *Neisseria*, and viruses such as human immunodeficiency virus) is *Chlamydia trachomatis* and its accompanying chlamydial species and animal host models. Development of a vaccine for humans against *Chlamydia* largely stems from global incident rates that remain high despite advancements in modern medicine.

The high incidence of new chlamydial infections is concerning from both a global health (e.g. 130 million new cases in 2012) (Newman et al. 2015) and financial perspective, even in developed countries where treatment is widely accessible (e.g. the annual cost of treatment of new chlamydial cases in the United States in 2008 was between 258 to 775 million USD) (Owusu-Edusei Jr et al. 2013). The incidence of *Chlamydia* (and likely facilitating transmission between seemingly healthy individuals) is partly due to high rates of subclinical infections (~80%). Chlamydial infections that progress to disease affect mucosal sites including the eyes, mucosa-draining lymph nodes, and urinary, respiratory, and

reproductive tracts (Brunham and Rey-Ladino 2005). When left untreated, genital serovars (genetically distinct strains of the same chlamydial species) can lead to pelvic inflammatory disease and more severe symptoms including infertility and ectopic pregnancy. Infection with ocular serovars of *C. trachomatis* can lead to scarring of the ocular conjunctiva potentially causing trachoma, the leading cause of preventable blindness in the world (Burton and Mabey 2009). In 2010, disability related to either sexually transmitted chlamydial diseases or trachoma resulted in approximately one million disability adjusted life years lost worldwide (Murray et al. 2012). Assuming these years are truly lost to disabled individuals missing a job at 20 hours a week while being paid 7.25 USD per hour (the U.S. federal minimum wage in 2018), approximately 7.5 trillion USD are lost from global chlamydia-related disability from new infections (conservatively estimated from 2010 incidence). Thus, the need for an effective *Chlamydia* prevention strategy for humans to reduce the incidence of chlamydial infections is a public health and economic benefit.

There are two main strategies for preventing new cases of chlamydial infection within a population: behavioural change (via sexual education) or individual immunological change (via vaccination; Gottlieb et al. 2014). In this study we focus on preventing chlamydial infections on the individual scale with novel (pre-clinical) chlamydial vaccines. From a therapeutic standpoint, chlamydial infections can be treated with antimicrobial compounds (e.g. azithromycin or doxycycline). These antimicrobial effects are relatively short lasting and can hinder the development of natural host immunity to future infections (Brunham and Rappuoli 2013). This is especially important as rates of reinfection can be as high as 25% in some populations, and reinfection is associated with an increased risk of disease sequelae (Hosenfeld et al. 2009). A chlamydial vaccine for humans is an ideal protective (and possibly

therapeutic) strategy that does not currently exist. Many excellent reviews have been published within the last 15 years regarding the development of such a vaccine (Brunham and Rappuoli 2013; Brunham and Rey-Ladino 2005; de la Maza et al. 2017; Farris and Morrison 2011; Longbottom and Livingstone 2006; Morrison and Caldwell 2002; Phillips et al. 2019; Rockey et al. 2009; Vasilevsky et al. 2014).

Testing of modern chlamydial vaccine candidates (i.e. immunizations) started in the late 1950s when Tang et al. described a technique to isolate *C. trachomatis* using chicken embryos (T'ang et al. 1957; Tang et al. 1958). Shortly thereafter in the early 1960s, an inactivated whole-cell immunization was developed in a similar manner to the polio vaccine and tested in a large-scale study that spanned several countries (Mabey et al. 2014). The results indicated that the inactivated whole-cell immunization elicited short term protection, yet some of the immunized individuals still developed trachoma. Since then, studies using mice, guinea pigs, koalas, pigs, and non-human primates have explored the immunogenicity of various *C. trachomatis* antigenic proteins and elucidated various aspects of the complex immunology surrounding chlamydial infection (Brunham and Rappuoli 2013; Brunham and Rey-Ladino 2005; Vasilevsky et al. 2014). Non-murine animal models were used to study chlamydial infection in biologically relevant hosts, including; *C. muridarum* infection in mice, *C. psittaci* infection in birds, *C. abortus* infection in livestock, *C. caviae* infection in guinea pigs, and *C. pecorum* infection in koalas. Advancements in modern molecular and computational techniques in recent years have led to an explosion in the number of published studies of chlamydial immunizations. This growing collection of empirical results, especially in the fields of chlamydial pathogenesis and vaccine development, makes identifying trends and advances in the field difficult, thus hindering

research progress. Indeed, many of these studies use different protocols (immunization formula, use of an adjuvant, immunization route, number of immunizations etc.) complicating between study comparisons (Phillips et al. 2019). The multitude of experimental variations can make identifying effective immunizations difficult, especially with the many ways of reporting common measurements (e.g. counting chlamydial infectious units or estimating chlamydial DNA to quantify the abundance of *Chlamydia*) and an objective unbiased assessment of the field is needed to facilitate ongoing advancements.

In this study, we conduct a meta-analysis from systematically selected literature specific to chlamydial immunizations and identify trends in the literature to focus research toward a chlamydial vaccine for humans. We recognize quality studies that may be highlighted by researchers in the field, but we chose to capture all studies in an unbiased meta-analysis to answer common questions from the field and identify strong research trends. From the published literature to date, we sought to answer the three questions previously raised: 1) Which chlamydial immunizations are most successful? 2) What are some of the emergent trends from successful chlamydial immunization studies using different animal hosts, often as models, for infection? And finally, 3) what effects do successful chlamydial immunizations have on the host immune system? To our knowledge, the use of meta-analyses to objectively evaluate vaccine development has not previously been employed and this study, therefore, represents an approach that could be used more widely for other pathogens subject to diverse vaccine development.

**2.3 Methods**

To begin, we sought to define what constitutes a successful chlamydial immunization. On the individual level, an ideal chlamydial vaccine will reduce disease sequelae, eliminate

infection, and elicit protection (as a strong anti-chlamydial host immune response) over an extended period of time. We understand there are many facets of a successful chlamydial vaccine, however we chose to define the success of an immunization based on the reduction of chlamydial organisms sampled from the host (i.e. chlamydial load). Chlamydial load is a relevant metric used to estimate the abundance of chlamydial organisms infecting a host.

*2.3.1 Literature search and study inclusion*

To identify relevant studies for the meta-analysis we first undertook a systematic search of the literature. This systematic search is reported according to Moher et al. (2009). On 08 Mar 2018 we searched the online citation database Web of Science to obtain relevant studies (across all years) published in English reporting the effects of chlamydial immunizations on the host immune system. We used the following search terms and Boolean operators:

TITLE: ((Chlamyd* AND (Vaccin* OR Immun*))) AND TOPIC: ((Vaccin*)) OR
TITLE: ((Trachoma* AND (Vaccin* OR Immun*))) AND TOPIC: ((Vaccin*))

These search terms were used to include studies that reported any variation of “chlamyd” (e.g. *Chlamydia* or chlamydial) and any variation of either “vaccin” (e.g. vaccination or vaccine) or “immun” (e.g. immunotherapy or immunization) in the title alone. As these search terms alone resulted in many irrelevant studies, an additional search term, “vaccin”, was used to obtain studies reporting any variation of this search term in either the abstract or keywords. Until the 1970s, many human chlamydial infections were reported as one of

their disease counterparts, trachoma (Grayston and Wang 1975). To include early immunization studies against trachoma, we added another set of search terms to include any variation of “trachoma” (e.g. trachoma or trachomatis) and either “vaccin” or “immun” in the title, and “vaccin” in either the abstract or keywords.

The resulting 390 studies (see Supplementary Figure 2.1) were downloaded to an EndNote Library (EndNote X8.2) and duplicates were removed. Two additional studies that were not captured by our search were added for full-text assessment. Studies were screened by abstract and were later processed for eligibility if the abstract described a chlamydial immunization and any quantitative measurement of either chlamydial load or a host immune parameter. Chlamydial immunizations were defined as being a modified or selected form (i.e. attenuated strains) of the chlamydial pathogen or component(s) of the pathogen intended to elicit a protective host immune response. For example, regardless of immunization route, studies using non-modified chlamydial organisms without adjuvant(s) (e.g. live unmodified *Chlamydia trachomatis* serovar D) were not included. Of the 392 studies initially screened, 230 were processed further by assessing the full text of each study. We defined vaccine success based on the protective effect of novel chlamydial vaccinations to reduce chlamydial loads, thus data were extracted only from studies by which vaccinations preceded a chlamydial challenge (i.e. the therapeutic effects of vaccines were not measured). Studies were included in the meta-analysis if the following information was included: 1) an immunized treatment group (defined previously), 2) a non-immunized control group (e.g. PBS or adjuvant only), 3) non-transgenic hosts (e.g. BALB/C mice or wild koalas), 4) a measurement of either chlamydial load or a host immune parameter (e.g. immune cell, cytokine, or antibody concentration), and 5) an acceptable form of reported

error with each measurement (95% confidence interval, standard error of the mean, standard deviation, interquartile range). Immune parameter measurements made in cell cultures without an immunized host (e.g. cytokine measurements using only immortal cell lines) were not used. Range is an unstable measure of variation and was not an acceptable form of error for our meta-analysis (Borenstein et al. 2009). Measurements without a specific quantification of immune parameters (e.g. gels without values) and histopathology measurements were excluded. Antibody measurements using complement fixation were excluded due to the variable sensitivity of this method (Bommana et al. 2017). Each full text assessment that we excluded from the meta-analysis was excluded with reasoning (see Supplementary Dataset 2.1).

*2.3.2 Data Extraction*

For each full text assessment used in our meta-analysis, we recorded the year, primary author, chlamydial species targeted by immunization, general host type (e.g. cats, birds, koalas, mice), chlamydial load or immune parameter, immunization type, immunization route, number of immunizations delivered, and control type. An additional column was made placing immunizations into broad categories to create a subset of the dataset for analysis: whole cell pathogens, multiple recombinant proteins (two or more antigenic chlamydial proteins delivered all at once, or two or more proteins delivered over the course of several single protein immunizations), single recombinant protein (hosts immunized with only one antigenic chlamydial protein during the duration of the experiment), anti-chlamydial antibodies, plasmid vectors expressing any chlamydial antigenic proteins, *Chlamydia* or chlamydial antigen exposed dendritic cells, viral vectors expressing any chlamydial antigenic proteins, or bacterial vectors expressing any chlamydial antigenic

proteins. More specific categories were made due to the abundance of whole cell pathogen (live virulent, dead virulent, or live attenuated) and single recombinant protein (CPAF, chlamydia protease activity factor; MOMP, major outer membrane protein; Pmp, peripheral membrane protein; or TTSS, type three secretion system) immunizations in our dataset. The mean, variance, and sample size for each chlamydial load or immune parameter measurement was recorded to calculate effect size. For each reported time point, each chlamydial load or immune parameter measurement was included in our dataset separately. In cases where multiple controls were used (e.g. hosts were separately immunized with either PBS or adjuvant only), we chose the control that most closely reflected the immunization given to the treatment group without containing chlamydial antigen (e.g. use of adjuvant only controls rather than PBS immunized controls when individuals in the treatment group were immunized with an antigen and an adjuvant). When multiple means were reported for individuals in a control or treatment group (e.g. separate mean IgG measurements for three individuals in a control group), mean values were pooled (2008). When multiple variances were reported where the sample size was given, variances were pooled using the equation described by the Cochrane Collaboration (2008) or were otherwise excluded if the sample size was unknown. When controls were not reported in the study as animals were difficult to obtain (e.g. non-human primates or koalas), we used explicitly defined as pre-immunization measurements (i.e. day 0) as controls. When the variance in a figure was not defined, we assumed the type of variance either by the variance defined in other figures within the study or the results from statistical tests. For studies with several measurements of diluted samples (e.g. IgG1 abundance), dilutions from other papers (e.g. Fairley et al. (Fairley et al. 2013) and Koroleva et al. 2017) measuring the same immune parameter were used and the two closest dilutions to other papers were included

(e.g. dilutions 1:400 and 1:800 were used for IgG1 measurements made by Bandholtz et al. 2002). When sample sizes were reported as a range, we used the average of that range. As mean measurements of chlamydial load or immune parameters are needed to calculate effect size, we used median values in our dataset in the absence of reported mean values in some instances (only when median values were reported, and a symmetrical distribution of the data could be assumed based on the variance reported as inter-quartile range; Higgins 2008).

*2.3.3 Effect size estimation and dataset preparation*

We estimated the effects of chlamydial immunizations from treatment groups with respect to a within-experiment control, creating a common metric to compare effect sizes between studies. An effect size is an index used to quantify a difference between two groups, in this case a treatment and a control group (Borenstein et al. 2009). We used an unbiased method of standardized estimation described by Hedges (Hedges 1981) that uses a correction factor to remove a bias where effect sizes calculated from small sample sizes are overestimated. To estimate effect size (g) and its variance ($V_g$), we used the following equations described by Borenstein et al. (2009):

$$g = d \cdot J \qquad V_g = V_d \cdot J^2$$

The standardized mean difference, *d*, is calculated as $d = \frac{x_c - x_t}{S_{within}}$ where $x_c$ is the mean value of the control measurement, $x_t$ is the mean value of the control measurement. $S_{within}$ is the within-groups standard deviation pooled across all groups calculated as $S_{within} = \sqrt{\frac{(n_c-1)\cdot {SD_c}^2 + (n_t-1)\cdot {SD_t}^2}{n_c+n_t-2}}$, where $SD_c$ is the standard deviation of the control group, $SD_t$ is the

standard deviation of the treatment group, $n_c$ is the sample size of the control group, and $n_t$ is the sample size of the treatment group. The variance of *d*, or $V_d$, is calculated as $V_d = \frac{n_c - n_t}{n_c \cdot n_t} + \frac{d^2}{2(n_c - n_t)}$. We used the common estimation for *J*, the correction factor for small sample sizes, by using the equation, $J = 1 - \frac{3}{4df - 1}$ where *df* represents degrees of freedom used to estimate $S_{within}$. To better interpret effect size, we used the inverse of g so that negative g values were associated with a decreased chlamydial load or immune parameter in treatment groups compared to control groups, and conversely, positive g values reflected an increased chlamydial load or immune parameter in treatment groups compared to control groups.

The type of error required to calculate effect size was standard deviation (SD), however different types were reported. When standard error of the mean (SEM) was reported, we estimated SD by multiplying SEM by the square root of the sample size. When interquartile range (IQR) was reported, we estimated SD by dividing IQR by 1.35 (2008). When a 95% confidence interval (95% CI) was reported, we estimated SD by multiplying the 95% CI by the square root of the sample size and dividing this by 1.96 (2008). A variance of "0" for both the control and treatment groups resulted in an error when calculating effect size by making $S_{within}$ equal to 0, thus making g and $V_g$ undefined. We replaced such values with "0.00001". We excluded 21 immune measurements as a result of this method due restrictions of our statistical software (R v3.4.3) being unable to compute effect sizes if the ratio of large to small sampling variance was too large. Data were cleaned such that log transformed mean and variance for some chlamydial load or immune parameter measurements that were reported as $\log_{10}$ or $\log_2$ transformed data were backwards transformed to obtain raw mean values for both control and treatment groups. We made

these transformations to make these measurements comparable with similar measurements reported as non-transformed data. For log transformed mean values, we made a backwards transformation of $\log_{10}$ or $\log_2$ values by raising the reported mean value to its base, 10 or 2, respectively (2008). The variance portion of the log transformed data was more complicated, requiring the log transformed SD to be converted into a log transformed upper 95% CI, then backwards transforming this to obtain a raw upper 95% CI, that was re-converted to obtain a raw SD.

*2.3.4 Analysis of effect size*

Summary effect size and variance were calculated using the MAd package using R v3.4.3 statistical software (Team 2017; Viechtbauer 2010). To analyze the effects of chlamydial immunizations on chlamydial load, we first created a subset of the data on host species (e.g. mice or pigs), then chlamydial species targeted by immunization (e.g. *C. muridarum* in mice or *C. trachomatis* in mice), then by comparisons that measured chlamydial load, and finally by immunization category. We performed a similar analysis for one of each of the following host immune parameters: IFNg, IgA, IgG, IgG1, IgG2a, IgG2b, and *in vitro* neutralization (IVN). Specific IgG isotypes were analyzed as independent categories and were separate from measurements of non-specified IgG (hereafter referred to as "IgG"). We created a subset of the data first by host species, then chlamydial species targeted by immunization, and finally by comparisons that measured one of the host immune parameters (each analysis performed separately). Once more we created a subset of this data by individual papers where a given immunization type (previously described) had both a chlamydial load measurement and a host immune parameter measurement. We used a meta-regression function from the MAd package to create omnibus linear models of our subset data, using

the previously calculated Hedge's g values for the response variable, and $V_g$ values for the variance. To better visualize the chlamydial load and host immune parameter data (see Figure 2.3), we performed a cube-root transformation on the mean effect size for all data represented. We used a Spearman's ranked correlation test to evaluate the relationship of average chlamydial load and average host immune parameter change (separated by chlamydial species, study, and immunization type) and set the level of significance at $p < 0.05$.

We recognize a publication bias might exist in the literature towards immunizations reporting chlamydial loads and host immune parameters. To test for publication bias we created funnel plots of standard error against effect size for chlamydial load, IFNg, IgA, IgG, IgG1, IgG2a, IgG2b, and IVN (Egger et al. 1997). Additionally, we conducted a weighted regression with multiplicative dispersion (with standard error as the predictor) to test for data distribution asymmetry as described by Sterne and Egger (Sterne and Egger 2005).

## 2.4 Results

### *2.4.1 Full dataset*

Our dataset includes 4,453 effect sizes for 165 studies that report chlamydial load or a measurement of a host immune response (See Supplementary Tables 2.1 and 2.2, and Supplementary Dataset 2.1 for included studies). A large portion of the dataset comprised of measurements of chlamydial load (1,424 comparisons, 110 studies, 32.0% of effect sizes in dataset). For quantitative measurements of the immunized host immune system (hereafter termed "immune parameter"), interferon gamma (IFNg) was reported in the greatest

number of studies (71), while the antibody immunoglobulin G (IgG) was measured the greatest number of times (resulting in 684 effect sizes).

Several hosts were used to test chlamydial immunizations. The following non-mouse hosts consisted of approximately 22.2% of our dataset (see Supplementary Table 2.3): koalas (8 studies, 355 effect sizes), pigs (6 studies, 268 effect sizes), birds (10 studies, 203 effect sizes), non-human primates (6 studies, 119 effect sizes), guinea pigs (2 studies, 15 effect sizes), cats (2 studies, 14 effect sizes), sheep (1 study, 10 effect sizes), and cows (1 study, 4 effect sizes). Not surprisingly, mice were the most common host model for chlamydial infection (129 studies resulting in 3,465 effect sizes or 77.8% of our entire dataset). The mouse model was used to investigate the effect of immunizations against *C. muridarum* (64 studies), *C. trachomatis* (45 studies), *C. abortus* (13 studies), *C. pneumoniae* (8 studies), *C. psittaci* (3 studies), or *C. pecorum* (1 study).

The advantages of studying chlamydial immunizations with mice as host models of infection are largely due to the known inbred pedigree of each mouse, availability of the murine immunological toolkit, and a smaller demand of veterinary resources compared to non-mouse models such as koalas or non-human primates. These advantages make mice ideal animal models for studying chlamydial immunizations and, not surprisingly, why the majority of the effect sizes in our dataset come from experiments using mice. There are limitations to mouse models of chlamydial infection that are used in experiments to develop a chlamydial vaccine for humans. In their review, Brunham and Ladino (Brunham and Rey-Ladino 2005) describe three major differences between *C. muridarum* infection in mice and *C. trachomatis* infection in humans: 1) mice can resolve *C. muridarum* infection in approximately 4 weeks, while humans can spontaneously resolve *C. trachomatis* infection

after several months, 2) *C. muridarum* and *C. trachomatis* have different immune evading strategies, (e.g. some *C. trachomatis* serovars can use pathways to biosynthesize tryptophan, while *C. muridarum* cannot), and finally 3) *C. trachomatis* has more allelic variation in MOMP (resulting in several serovars) compared to *C. muridarum* (only one serovar). Mice can be infected with *C. trachomatis*, but are otherwise poor hosts due to the high dosage of *C. trachomatis* necessary for infection and a short resolution time (about 2-3 weeks) thereafter. These are important distinctions, and we chose to compare immunizations against *C. muridarum* and *C. trachomatis* in mice as this data was the most abundant. We discuss the results from non-mouse studies in the Supplementary text (see Supplementary Text 2.1 and Supplementary Table 2.4) using a finer scope than that used to analyze studies using the mouse model. There exist many important aspects of chlamydial immunization experiments that were included in our dataset that were not included in our meta-analysis such as: adjuvant, delivery route, sampling date (days post challenge), and sampling type. We split our dataset by chlamydial species, host type, and immunization type and, despite not being able to include all sources of experimental variation, we were able to detect important patterns at this level.

### *2.4.2 Mouse immunizations against Chlamydia muridarum*

*Whole cell immunizations*. Both dead and live virulent strains on average were effective at reducing chlamydial load (see Figure 2.1a and Table 2.1). Non-modified *Chlamydia* (i.e. live virulent strains) were only included in our analysis if they were delivered with an adjuvant (see Methods). Live virulent immunizations with an adjuvant from 3 studies reduced *C. muridarum* load in mice (-0.427 ± 0.205; Table 2.1), while dead virulent strains from 9 studies on average had a greater reduction on *C. muridarum* load (-1.328 ± 0.745). When

considering all studies of whole cell *C. muridarum* immunizations, our assessment suggests that dead virulent strains (often heat killed or irradiated) were on average more effective immunizations compared to live virulent strains with an adjuvant. Though excluded from our analysis, live virulent strains without an adjuvant have been previously shown to reduce chlamydial load (and are often used as positive controls), but their use as a vaccine is problematic due to their propensity to result in reproductive disease (Lu et al. 2012; Vasilevsky et al. 2014).

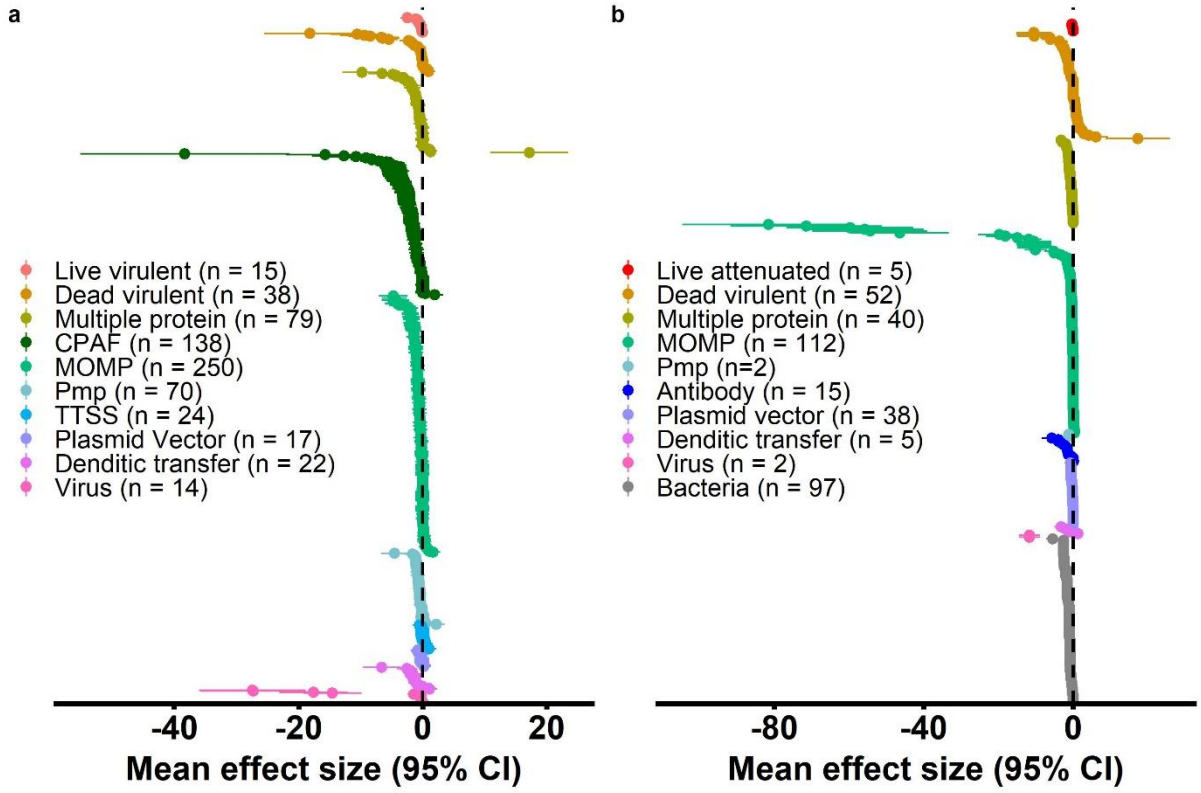



**Figure 2.1.** Many chlamydial immunizations (with *n* effect sizes) reduced chlamydial load against *C. muridarum* (a), and C. *trachomatis* (b) in mice. Each dot represents a calculated Hedge's g effect size (± 95%CI). For average effect size of each immunization category, see Table 2.1. Negative effect sizes with error that do not overlap 0 (indicated by the dotted line) have a negative effect on chlamydial load. Immunization groups that have the greatest negative effect on chlamydial load will have the majority of their values below 0 (the effect threshold). Immunizations were placed in the following groups: whole pathogens (live virulent with adjuvant, dead virulent, live attenuated), multiple proteins (two or more native or recombinant proteins delivered all at once or two or more proteins delivered over the course of several single protein immunizations), single proteins (native or recombinant; CPAF, MOMP, Pmp, TTSS), anti-chlamydial antibodies, plasmid vectors expressing any chlamydial antigenic proteins, *Chlamydia* or chlamydial antigen exposed dendritic cells, viral vectors expressing any chlamydial antigenic proteins, and bacterial vectors expressing any chlamydial antigenic proteins. CPAF = chlamydia protease activity factor, MOMP = major outer membrane protein, Pmp = peripheral membrane protein, TTSS = type three secretion system.

**Table 2.1.** Estimate (i.e. average) of change in chlamydial load from the meta-regression, variation (lower and upper confidence intervals), and sample size for chlamydial immunizations against *C. muridarum* and *C. trachomatis* in mice. Estimates with averages below 0 have a negative effect on chlamydial load (i.e. data from the literature support these immunizations reducing chlamydial load). See Figure 2.1 caption for an explanation of immunization groups.

| Species | Immunization | | Estimate (2.5%, 97.5% CI) | Effect sizes | Studies |
|---|---|---|---|---|---|
| *C. muridarum* | Whole pathogen | Live virulent | -0.427 (-0.632, -0.222) | 15 | 3 |
| | | Dead virulent | -1.328 (-2.073, -0.584) | 38 | 9 |
| | Multiple proteins | | -0.823 (-1.097, -0.549) | 79 | 6 |
| | Single protein | CPAF | -1.833 (-2.098, -1.567) | 138 | 8 |
| | | MOMP | -0.553 (-0.653, -0.453) | 250 | 25 |
| | | Pmp | -0.535 (-0.651, -0.420) | 70 | 6 |
| | | TTSS | 0.169 (-0.061, 0.400) | 24 | 1 |
| | Plasmid expression vector | | -0.261 (-0.526, 0.004) | 17 | 4 |
| | Dendritic cell adoptive transfer | | -1.186 (-1.610, -0.761) | 22 | 6 |
| | Viral vector | | -5.770 (-10.823, -0.718) | 14 | 2 |
| *C. trachomatis* | Whole pathogen | Live attenuated | -0.150 (-0.374, 0.074) | 5 | 1 |
| | | Dead virulent | -0.461 (-0.995, 0.072) | 64 | 3 |
| | Multiple proteins | | -0.755 (-0.938, -0.573) | 50 | 8 |
| | Single protein | MOMP | -0.413 (-0.521, -0.305) | 125 | 11 |
| | | Pmp | -1.136 (-1.780, -0.492) | 2 | 1 |
| | Antibody | | -1.676 (-2.410, -0.942) | 15 | 1 |
| | Plasmid expression vector | | -0.240 (-0.378, -0.102) | 38 | 5 |
| | Dendritic cell adoptive transfer | | -1.270 (-2.705, 0.165) | 5 | 1 |
| | Viral vector | | -11.693 (-13.655, -9.730) | 2 | 1 |
| | Bacterial vector | | -1.210 (-1.355, -1.065) | 97 | 5 |

CPAF = chlamydia protease activity factor, MOMP = major outer membrane protein, Pmp = peripheral membrane protein, TTSS = type three secretion system

*Recombinant and natural protein immunizations*. Single recombinant or native protein immunizations against *C. muridarum* formed a quarter of our dataset (27.9%; 1,206 effect sizes). Four proteins were commonly used in single protein chlamydial immunizations: CPAF (chlamydial protease activity factor), MOMP (major outer membrane protein), Pmp (peripheral membrane protein), and TTSS (type three secretion system). CPAF had the greatest effect on chlamydial load compared to the other single protein immunizations (-1.833 ± 0.265). Both MOMP and Pmp had similar effect sizes reducing chlamydial load (-0.553 ± 0.100, and -0.535 ± 0.116, respectively). Surprisingly, the proteins associated with TTSS (both the needle and its tip) were not effective antigens in chlamydial immunizations (0.169 ± 0.231). Multiple protein immunizations increase efficacy (-0.823 ± 0.274) only slightly more than single MOMP or Pmp immunizations (by comparison of mean effect sizes). The addition of multiple proteins may affect host immune responsiveness to multiple chlamydial serovars which was not included in our analysis.

*Viral, dendritic cell adoptive transfer, and plasmid expression vector immunizations.* On average viral vector immunizations (see Figure 2.1) had a strong effect that reduced chlamydial load (-5.770 ± 5.053), however there were only two studies that investigated the use of immunizations with an antigenic chlamydial component expressed on a viral vector against *C. muridarum*. Despite challenges harvesting and producing dendritic cells on a small scale (Jiang et al. 2008), the data suggest dendritic cell adoptive transfer immunizations seem to be effective at reducing chlamydial load (-1.186 ± 0.424). Immunizations that use chlamydial DNA (either entire genes or fragments) and an antigenic plasmid vector had no effect on chlamydial load as the variance (95% confidence interval) crosses 0, or the threshold of effect (-0.261 ± 0.265).

*2.4.3 Mouse immunizations against Chlamydia trachomatis*

*Whole cell immunizations.* Two whole cell immunizations were used in mice against *C. trachomatis*: dead virulent strains, and live attenuated strains (see Figure 2.1b and Table 2.1). We included 4 studies of whole cell immunizations against *C. trachomatis* in our meta-analysis (see Table 2.1). Both immunizations had error (95% CI) spanning "0", the threshold of effect as live attenuated immunizations had an effect size of -0.150 (± 0.224), and dead virulent immunizations had an effect size of -0.461 (± 0.534). Our meta-analysis indicates that there was a large amount of variation for whole cell immunizations against *C. trachomatis* across all studies, and this immunization type did not have a strong effect on chlamydial load. Indeed, Stary et al. (2015) show that the efficacy of dead virulent immunizations in reducing chlamydial load are largely dependent on immunization route and adjuvant type.

*Viral, bacterial, dendritic cell adoptive transfer, antibody and plasmid expression vector immunizations.* Only two studies using viral vector immunizations were included in our dataset, yielding promising results and having a negative effect on chlamydial load (-11.693 ± 1.962). More work is needed to determine the efficacy of these immunizations against *C. trachomatis*. Bacterial vectors (e.g. vibrio cholera ghosts) expressing chlamydial antigens were used in 5 studies which were largely effective (-1.210 ± 0.145). Dendritic cell adoptive transfer, which was effective against *C. muridarum*, had a large variance and no clear effect on chlamydial load (-1.270 ± 1.435). On average, anti-chlamydial antibodies reduced chlamydial load, though only one study (Whittum-Hudson et al. 1996) used this immunization type in our dataset (-1.676 ± 0.734). Interestingly, plasmid expression vector

immunizations in the mouse model (which had no effect against *C. muridarum*) had a small negative effect against *C. trachomatis* (-0.240 ± 0.138).

*Recombinant and natural protein immunizations.* MOMP and Pmp were the only single protein (native or recombinant) immunizations to be used against *C. trachomatis*, that were included in our analysis. On average MOMP was effective at reducing chlamydial load (-0.413 ± 0.108) and was equally efficacious when comparing *C. trachomatis* and *C. muridarum* MOMP immunizations. Pmp immunizations were effective at reducing chlamydial load, but were less commonly studied (-1.136 ± 0.644, n = 1 study). Adding multiple proteins to an immunization had a negative effect on chlamydial load, and seemed to slightly increase efficacy compared to single MOMP immunizations (-0.755 ± 0.183).

### *2.4.4 Chlamydial immunized host bioprofile*

*Bioprofile data interpretation.* In Figure 2.2a we show possible outcomes of graphing data from individual studies that measured both a host immune parameter and chlamydial load so as to provide a conceptual guide to the reader. One might expect a "good" immunization to negatively affect chlamydial load and positively affect anti-chlamydial host immune parameters. Such immunizations would result in studies being plotted in the upper left quadrant. As most immunizations reduced chlamydial load and increased immune parameters (see above for chlamydial load effect sizes), conceptual interpretations for data in this quadrant are given. Figure 2.2b illustrates two possible outcomes that could occur when plotting a line made from a regression: 1) hosts with a greater magnitude reduction on chlamydial load tend to stimulate a greater host immune parameter (blue line, negative slope), and 2) hosts with a greater reduction in chlamydial load tend to have no effect (or a less obvious effect) on a host immune parameter (red line, slope close to 0). We tested

whether the magnitude of host immune parameter and chlamydial load change exists using a Spearman's correlation where negative correlation coefficients support a negative relationship (blue line, Figure 2.2b).

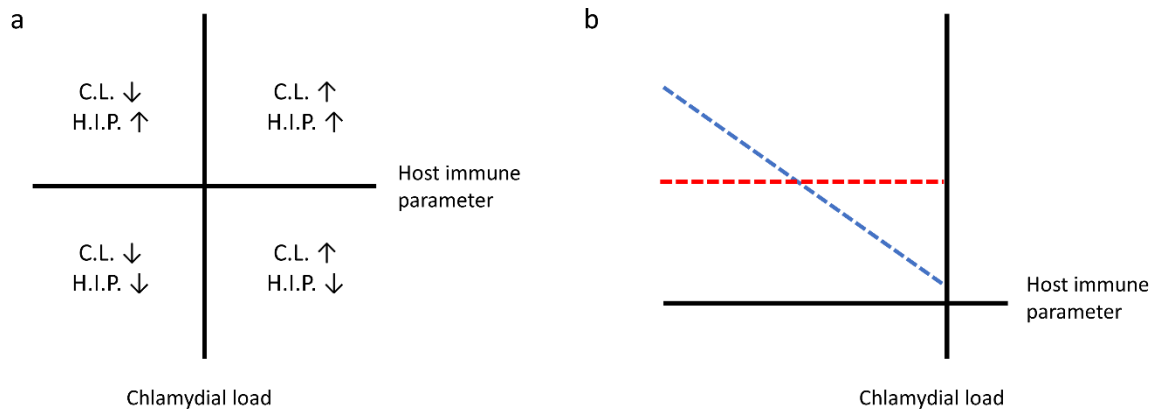



**Figure 2.2.** Possible outcomes of chlamydial load plotted against host immune parameter given hypothetical data plotted in each quadrant (a), and two hypothetical trendlines for immunizations resulting in decreased chlamydial load and increased host immune parameters (b). The threshold for immunizations that affect chlamydial load is x=0, while the threshold for a given host immune parameter is y=0 (i.e. effect sizes below x=0 have a negative effect on chlamydial load, and y=0 have a negative effect on host immune parameter). The colored lines (in panel b) represent two possible trends in which there was a general reduction in chlamydial load: blue, a greater reduction in chlamydial load (more negative on the x axis) is associated with a large increase in a host immune parameter (more positive on the y axis); and red, a greater reduction in chlamydial load is unrelated to increases in a host immune parameter.

*Realized host bioprofile.* More than half of the measured host immune data in our dataset were from mice immunized against either *C. muridarum* or *C. trachomatis* (collectively 58.9%, 1,739 effect sizes). Extracted data from measurements of mice immunized against these two chlamydial species mainly consisted of the following immune parameters: IFNg, IgA, IgG (unspecified isotype), IgG1, IgG2a, IgG2b, and *in vitro* neutralization (IVN; see Supplementary Table 2.2 for number of effect sizes and studies, and Supplementary Dataset 2.2 for effect size values and variance).

In Figure 2.3, we show general increases in immune parameters for most immunizations against *C. muridarum* and *C. trachomatis*. Most of the data occurred in the top left quadrant, indicating that chlamydial immunizations from most studies resulted in decreased chlamydial load (see Results 2.4.2 and 2.4.3) and increased IFNg, IgA, IgG1 and IgG2a antibodies. Studies reporting anti-*C. trachomatis* IgG (unspecified isotype) also followed this pattern (see Supplementary Figure 2.2). With three exceptions, the linear regressions in Figure 2.3 indicate a clear negative relationship between studies reporting mean chlamydial load and mean host immune parameter change (i.e. blue line in Figure 2.2b). Anti-*C. trachomatis* IgG1 has a slightly negative relationship that is limited to only 5 studies that were included in this analysis. Lastly, there was a negative relationship between IFNg and *C. trachomatis* chlamydial load, although only when an outlier study (Igietseme and Murdin 2000) was omitted (see black dashed line, Figure 2.3b). When analyzing all studies reporting IFNg in our meta-analysis, we found no clear relationship between average changes to host IFNg and *C. muridarum* chlamydial load (i.e. red line in Figure 2.2b).

Table 2.2 shows all of the relationships of the magnitude of mean chlamydial load and mean host immune parameters (see Table 2.2). With one exception (unspecified isotype IgG, see Table 2.2), all host immune parameters had a negative Spearman's rank correlation coefficient indicating that greater chlamydial load decreases were associated with large increases in host immune parameter. This relationship was statistically significant ($p<0.05$) for studies immunizing against *C. muridarum* reporting anti-chlamydial IgA, IgG1, and IgG2a antibodies. Studies immunizing against *C. trachomatis* reporting anti-chlamydial IgA and IgG2a antibodies were also statistically significant. When considering all studies, measurements of average IFNg in hosts immunized against *C. muridarum* were not correlated with average chlamydial load change ($p = 0.414$), and studies immunizing against *C. trachomatis* were trending ($p = 0.058$).

### *2.4.5 Publication bias*

We chose to look at whether a bias exists in the literature to determine whether the effect sizes we obtained from our meta-analysis were possibly influenced by publication bias. Plotting effect size against standard error for all chlamydial load or host immune parameter data extracted from the literature results in funnel plots that appear asymmetrically distributed (see Figure 2.4). To investigate this further, we conducted an Egger's regression for funnel plot asymmetry for each variable with standard error as a predictor. Regressions for chlamydial load ($t = -24.77$, $p<0.0001$), IFNg ($t = 13.50$, $p<0.0001$), IgA ($t = 26.26$, $p<0.0001$), IgG ($t = 30.16$, $p<0.0001$), IgG1 ($t = 13.14$, $p<0.0001$), IgG2a ($t = 32.764$, $p<0.0001$), IgG2b ($t = 14.435$, $p<0.0001$), and IVN ($t = 6.78$, $p<0.0001$) further suggest a bias exists (Team 2017).

**Table 2.2.** Results of a Spearman's ranked correlation for host immune parameter and chlamydial load measurements in individual studies after immunization against either *C. muridarum* or *C. trachomatis*. Average values of the host immune parameters interferon gamma (IFNg), immunoglobulin A (IgA), IgG, IgG1, IgG2a, IgG2b, and in vitro neutralization (IVN) were estimated from individual studies and paired with the average chlamydial load effect size from the same study. The resulting correlation coefficient, p-value, number of immunizations and number of studies are shown. Bold p-values indicate significant correlations ($p < 0.05$).

| Species | Host immune parameter | Correlation coefficient (Spearman's rho) | p-value | Number of immunizations* | Number of studies |
|---|---|---|---|---|---|
| *C. muridarum* | IFNg | -0.157 | 0.414 | 29 | 25 |
| | IgA | -0.564 | **0.031** | 15 | 12 |
| | IgG | 0.536 | 0.236 | 7 | 5 |
| | IgG1 | -0.530 | **0.012** | 22 | 18 |
| | IgG2a | -0.563 | **0.007** | 22 | 18 |
| | IgG2b | -0.036 | 0.964 | 7 | 7 |
| | IVN | -0.600 | 0.350 | 5 | 4 |
| *C. trachomatis* | IFNg | -0.521 | 0.058 | 15 | 14 |
| | IgA | -0.527 | **0.032** | 17 | 15 |
| | IgG | -0.476 | 0.121 | 12 | 8 |
| | IgG1 | -0.700 | 0.233 | 5 | 5 |
| | IgG2a | -0.850 | **0.006** | 9 | 9 |
| | IgG2b | -1.000 | 1.000 | 2 | 2 |
| | IVN | N/A | N/A | 1 | 1 |

*contains one or more immunizations from each study with an effect size on both chlamydial load and a host immune parameter. Immunizations were grouped into previously described immunization types and then averaged.

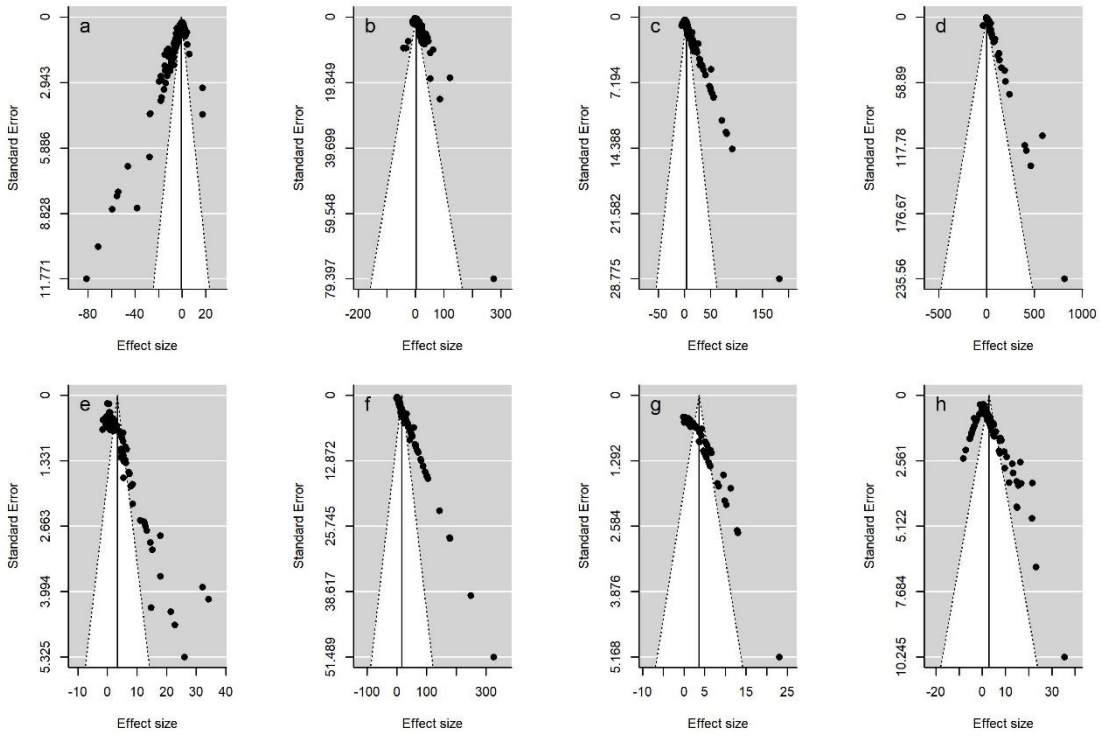



**Figure 2.4.** Funnel plots showing the distribution of all effect sizes against their respective standard errors for chlamydial load (a), interferon gamma (IFNg; b) Immunoglobulin A (IgA; c), IgG (d), IgG1 (e), IgG2a (f), IgG2b (g) and in vitro neutralization (IVN; h). Funnel plots are a graphical representation of the dispersion of effect sizes around 0. The vertical black line indicates the mean effect size of all points for each plot and the dashed lines indicate pseudo 95% confidence limits for heterogeneity. Publication bias is possible as the majority of values are asymmetrically distributed in the direction of negative results for chlamydial load (i.e. reductions in chlamydial load), and positive results for IFNg, IgA, IgG, IgG1, IgG2a, IgG2b, and IVN (i.e. increases in host immune parameters) after immunization.

## 2.5 Discussion

Many expanding fields of microbiology research, including chlamydial vaccine development, contain extensive empirical data published annually (where each study reports different protocols) making it difficult to objectively identify progress in the area. Meta-analysis is a useful tool to synthesize these results and has previously been used to determine vaccination efficacy. These reviews usually report vaccination efficacy as risk ratios, odds ratios, or risk differences (sometimes called treatment effects) and are restricted to binary data such as pathogen or disease prevalence between vaccinated and control groups. Going beyond binary data, an estimation of effect size described by Hedges (Hedges 1981) (sometimes called Hedges g) uses quantitative measurements extracted from the literature to compare the effect of a treatment group to a control group within the same experiment. This method has already been used in fields outside of microbiology such as ecology and social sciences (Hofmann et al. 2010; Lunn et al. 2017). In the current study, we used Hedges method of estimating effect size to identify efficacious chlamydial immunizations (placed in broad categories) and their effects on commonly measured host immune parameters.

### *2.5.1 Successful chlamydial immunizations*

The majority of chlamydial research has been conducted in murine hosts, making this host species a focal point of our meta-analysis. With the exception of one immunization group (single protein TTSS immunizations against *C. muridarum*), the average effect size of all murine immunization groups against *C. muridarum* and *C. trachomatis* reduced chlamydial load. As there are multiple promising immunization categories tested against these two chlamydial species, we created criterion to guide our interpretation of which immunizations were most successful at reducing chlamydial load. We chose grouped immunizations (listed

in Table 2.1) that resulted in an average estimated effect size less than -1 with both confidence intervals below 0. Additionally, we chose immunization groups that had been published in more than one study with a replication threshold of at least 30 effect sizes. Using these criterion, chlamydial protease activity factor (delivered as a single protein; CPAF) and dead/inactivated virulent whole cell immunizations were most effective at reducing *C. muridarum* load in mice (see Figure 2.1 and Tables 2.1 and 2.2). An overwhelming majority of CPAF immunizations were delivered intranasally (118 of 138 effect sizes) while half of the dead/inactivated virulent *C. muridarum* immunizations were delivered intranasally (19 of 38 effect sizes). The importance of mucosal immunizations has been shown by Stary et al. (2015) who immunized mice against *C. trachomatis* via the mucosa, resulting in the recruitment of antigen specific T cells to mucosal sites and systemic circulation.

While MOMP immunizations are considered the most promising chlamydial immunizations in many studies, our meta-analysis combining results from 165 studies identified CPAF as a promising immunization for the reduction of *C. muridarum* load in mice. CPAF is a chlamydial protease secreted in the host cytosol responsible for the degradation of host transcription factors responsible for major histocompatibility complex (MHC) gene activation that is highly stable in the host cell cytoplasm with a relatively low degradation rate (Shaw et al. 2002; Zhong et al. 2001). Two main concerns were raised over CPAF immunizations: 1) CPAF immunizations have the potential for MHC interference after immunization (Longbottom and Livingstone 2006), and 2) CPAF immunizations have the potential for pathogenesis, particularly the development of oviduct pathology (Vasilevsky et al. 2014). Studies have addressed these concerns, particularly Chaganty et al. (Chaganty et

al. 2010) and Li et al. (2007) with evidence supporting CPAF immunizations. Additionally, Tan and Sütterlin (2014) were unable to detect the degradation of several CPAF targets. When comparing CPAF and MOMP immunizations against *C. muridarum*, our meta-analysis conservatively indicates (comparing the lower 95% CI of CPAF and the upper 95% CI of MOMP against *C. muridarum*) that the effect size of CPAF immunizations are approximately twice as effective at reducing chlamydial load to MOMP immunizations.

Using the previously mentioned criterion, bacterial vector immunizations were the most effective immunizations against *C. trachomatis* in mice. It was surprising that single protein MOMP immunizations were less effective compared to bacterial immunizations against *C. trachomatis* as MOMP in general has long been regarded as a promising vaccine candidate after its characterization over 30 years ago (Caldwell et al. 1981). MOMP is an external protein on infectious EBs covering approximately 60% of its outer membrane complex and functions as a porin (Nikaido 2003; Sun et al. 2007). We found studies of immunizations against all chlamydial species and in all animal hosts where MOMP was tested alone or as a component of a multiple protein immunization, highlighting its importance (see Supplementary Table 2.4 and Supplementary Text 2.1). When comparing all studies of MOMP immunizations against *C. trachomatis* and *C. muridarum*, we found the two to be comparable. The majority of MOMP immunizations against *C. trachomatis* in mice were delivered subcutaneously (80 of 125 effect sizes), while the majority of bacterial immunizations were delivered intramuscularly (62 of 97 effect sizes). An interesting dichotomy exists among delivery routes of *C. trachomatis* and *C. muridarum* immunizations suggesting that non-mucosal routes are still being studied despite what is known about mucosal protection against chlamydial infection. This could be due to the difficulty of

immunizing hosts intranasally multiple times as opposed to intramuscular or subcutaneous delivery of chlamydial immunizations which is common among widely used vaccines such as those against human papillomavirus (HPV), influenza, or hepatitis B.

*2.5.2 Host bioprofile after chlamydial immunization*

We selected key anti-chlamydial host immune parameters commonly measured in murine hosts immunized against *C. trachomatis* and *C. muridarum*: interferon gamma (IFNg), immunoglobulin A (IgA), IgG, IgG1, IgG2a, IgG2b, and *in vitro* neutralization (IVN). Our meta-analysis across all studies showed most chlamydial immunizations reduced chlamydial load and increased each of these immune parameters, agreeing with the biological consensus that a combination of humoral and cell mediated immune responses reduce chlamydial load (Farris and Morrison 2011; Hafner et al. 2008). IFNg is arguably the most important host cytokine in response to a chlamydial infection. IFNg reduces the concentration of tryptophan (necessary for the growth some chlamydial species) by inducing the expression of indoleamine-2,3-dioxygenase, resulting in tryptophan catabolism (Brunham and Rey-Ladino 2005; de la Maza et al. 2017; Longbottom and Livingstone 2006; Phillips et al. 2019; Vasilevsky et al. 2014). Multiple studies show IFNg mediated tryptophan starvation reduces growth (and in some cases leads to persistence) of some chlamydial species *in vitro* (Beatty et al. 1994; Beatty et al. 1993; Leonhardt et al. 2007). Across all studies, we found a trending relationship between average IFNg increase and *C. trachomatis* decrease, while a relationship between the magnitude of IFNg increase and *C. muridarum* decrease was less obvious. This could be due to a number of factors but is most likely to be caused by the difficulty of single measurements (i.e. at one or two timepoints) of IFNg *ex vivo*. More specifically, several measurements of IFNg after immunization against *C. muridarum*

resulted in lower IFNg in immunized mice compared to control mice ten days post challenge (Cheng et al. 2009; Cheng et al. 2014; Pal et al. 2017a; Pal et al. 2017b). Cheng et al. attribute this to chlamydial infection resolution where high levels of IFNg may no longer be necessary (Cheng et al. 2014). We included these measurements in our meta-analysis (such IFNg effect sizes were negative), thus making the relationship between IFNg and chlamydial load change less obvious when all studies were considered.

The production of anti-chlamydial antibodies in response to chlamydial infection has been discussed and investigated since the early testing of modern chlamydial immunizations in the 1960s (Chang et al. 1964; Collier and Blyth 1966). An increase in anti-*Chlamydia* antibodies has been negatively correlated with chlamydial load through a number of mechanisms proposed (e.g. opsonization, antibody dependent cellular cytotoxicity, or complement activation) (Bulir et al. 2016; de la Maza et al. 2017). Indeed, mechanisms of chlamydial reduction as measured by serum neutralization assays is dependent on antibody function (i.e. recognition or neutralization). In our meta-analysis, we found both IgA and IgG2a antibody increase correlated with chlamydial load decrease for mice immunized against either *C. muridarum* or *C. trachomatis*. Additionally, an increase in IgG1 was correlated with *C. muridarum* load reduction (18 studies), but not *C. trachomatis* load reduction (5 studies). More studies measuring IgG1, IgG2b, or IVN may make these relationships clearer. Lastly, our analysis shows that systemic IgG is the most common antibody measured for *Chlamydia* immunization studies. Previous work has shown that mucosal IgA antibodies, and not systemic IgG, are likely to be a more relevant marker of chlamydial protection (Brunham et al. 1983). Future studies should consider measurements

of mucosal immune parameters when determining the effects of chlamydial immunizations on host protection.

### *2.5.3 Chlamydial vaccine development publication bias*

We found publications of chlamydial immunizations to be highly bias toward the reporting of negative chlamydial load measurements and positive host immune parameters measurements in treatment groups relative to control groups. Though the mechanism for this bias is unclear, the effect on published chlamydial vaccines is presumed to be detrimental. Egger et al. (Egger et al. 1997) describe several sources for funnel asymmetry (see Figure 2.4). It may be possible that more efficacious immunizations may be cited more frequently among researchers, while less efficacious immunizations are less frequently cited and are therefore less often published. Egger et al. (Egger et al. 1997) suggest that a citation bias is likely to negatively affect smaller studies that have a lower publication priority compared to larger studies that may garner more citations. It is unknown how the inclusion of unpublished studies would affect the results of our meta-analysis.

Unpublished studies, especially those with negative results, limit vaccine development and hinder research progress. Access to a repository of negative results is valuable, where the reporting of quality negative results can lead to a more precise understanding of immunization effectiveness against chlamydial infection. At present, the *Journal of Negative Results in Biomedicine* is no longer accepting manuscripts for peer review, however PLoS ONE currently considers studies with negative results. We urge researchers and publishers to report and publish negative results for quality experiments testing immunizations against *Chlamydia* or other pathogens of interest.

*2.5.4 Direction of chlamydial vaccine development*

Here we described a method used to evaluate the literature to guide future studies toward an eventual chlamydial vaccine for humans. We defined a successful immunization as a reduction in chlamydial load after immunization. Chlamydial load is a quantifiable measurement used to estimate the abundance of chlamydial organisms infecting a host that is reported in the majority of studies included in our meta-analysis. This measurement is quantifiable regardless of host development of chlamydial related diseases and is the most consistent measurement across chlamydial vaccine studies. Pathogenesis of chlamydial disease is highly complex and affected by multiple environmental and genetic factors and is an important facet of chlamydial infection (Darville and Hiltke 2010). When considering whether an immunization is successful, especially one incorporating live whole cell pathogen, the propensity to cause *Chlamydia*-related disease should be considered.

There exist multiple challenges to developing a human vaccine against *C. trachomatis*. Though many more exist, three major challenges are 1) the difficulty in eliciting immune responses resulting in protection against chlamydial infection and disease using different combinations of chlamydial antigens, adjuvants, and delivery routes, 2) overcoming chlamydial evasion and persistence responses, and 3) protecting against multiple chlamydial serovars. Despite the obstacles before chlamydial vaccine development, a human vaccine within the next decade is possible (de la Maza et al. 2017). There exist two major sources of evidence for vaccine development, each with their own limitations. One major source of evidence are the observational studies of chlamydial infections in humans. Studies publishing human immune responses to chlamydial genital infection are important but limited by ethical concerns and opportunistic measurements that are less important for

chlamydial infection (e.g. serum antibodies) (Sharma et al. 2004; Sharma et al. 2005). The second major source of evidence is the experimental testing of vaccines using non-human models of infection. Inbred non-human animal models of infection allow for controlled experiments, but are unable to introduce some complex aspects of chlamydial infections of outbred human hosts (e.g. host genetics, and sexual behavior). Indeed, researchers need to consider critical differences in the immune system between humans and non-human animals, such as birds having IgY instead of IgG (Spillner et al. 2012). Despite where the evidence originates, concerns have been made about how to progress the development of a chlamydial vaccine beyond research laboratories. Starnbach (Starnbach 2018) suggests that advancing the development of a chlamydial vaccine requires the resources of a large pharmaceutical company with government funding to safely and ethically test chlamydial vaccines in humans.

Recently, one chlamydial vaccine candidate for humans consisting of CTH522 completed a phase I trial (Abraham et al. 2019). Described by Olsen et al. (2015), CTH522 is a fusion of recombinant MOMP (serovar D) and Hirep, a fusion of MOMP variable domain epitopes with conserved membrane anchors from multiple serovars (D, E, F, and G). Substantial work from this group developed CTH522 in a number of experiments using both mice and pigs as host models (Boje et al. 2016; Kuczkowska et al. 2017; Lorenzen et al. 2015; Olsen et al. 2015; Olsen et al. 2017; Wern et al. 2017). Using CTH522 as an example of a successful preclinical immunization, how effective are animal models as hosts of infection used to establish effective proteins to be used in clinical trials? Data from our meta-analysis indicate that CTH522 and Hirep immunizations (Hirep1 and 2) are successful at reducing chlamydial load in mice against *C. trachomatis* infection. The mean effect sizes were -0.348 and -0.468

for Hirep and CTH522, respectively, and were comparable to the average effect size of MOMP immunizations in mice against *C. trachomatis* listed in Table 2.1. Hirep immunizations in mice, on average, had a positive effect on IFNg (1.360), IgA (0.551), and IgG (isotype not specified; 6.182). In the pig hosts, the reduction in chlamydial load was negatively trending with large variances in effect size (see Supplementary Table 2.4). Indeed, the success of CTH522 progressing beyond the preclinical stage highlights the importance of protection against multiple serovars in immunizations.

### *2.5.5 Applicability to fields outside of chlamydial vaccine development*

In this study, we have synthesized thousands of fragmented results from studies of chlamydial vaccine development under a single, comparable, unbiased, set of analyses. This approach can be used in fields where the number of studies of new preclinical immunizations are growing. Vaccine development, especially against sexually transmitted infections have a growing collection of quantitative data. Similar to our previous search terms and Boolean operators for our chlamydial immunization search, we searched for studies of immunizations against herpes simplex virus type 1, *Trichomonas vaginalis*, *Treponema pallidum*, and *Neisseria gonorrhoeae* (see Supplementary Table 2.5). This search resulted in 110 scientific articles for herpes simplex virus type 1, 17 for *T. vaginalis*, 50 for *T. pallidum*, and 47 for *N. gonorrhoeae*. Fewer studies of immunizations against these sexually transmitted pathogens exist compared to our search for chlamydial immunizations where we found 389 studies (before screening studies). The number of immunization studies are likely to increase as preclinical trials benefit from technological advances, such as transgenic "humanized" mice. For example, Gottlieb et al. (2016) suggest that the biggest hurdle for *N. gonorrhoeae* vaccine development is due to the difficulty in challenging laboratory hosts

(particularly mice) and humanizing murine hosts might be key. From our Web of Science search, we identified 17 *N. gonorrhoeae* immunization studies from the start of 2016 or 36% of the total number of studies in just over three years. Indeed, estimations of effect size such as the one used here for chlamydial immunizations are going to be beneficial to identifying trends in growing fields.

Estimates of effect size are critical to identifying trends in vaccine development where the number of publications and the quantity of empirical results have grown too large to be easily interpretable. A large body of research exists for the development of a vaccine for humans against human immunodeficiency virus. After a search in Web of Science for studies for the development of a human immunodeficiency virus vaccine (see Supplementary Table 2.5), we identified 1,638 studies. A review of papers published in 2018 by Burton (Burton 2019), focuses on immunizations that elicit an immune response of broadly neutralizing antibodies due to their protective capabilities against HIV infection. With the methods described here, one could estimate the effect of HIV immunizations on broadly neutralizing antibodies or any other relevant host immune parameters found in the literature.

Lastly, estimates of effect size may be useful for vaccine development when optimizing vaccines that currently exist. For example, one vaccine exists within the field of chlamydial vaccine development, a vaccine against *C. abortus* for livestock made from an attenuated mutant strain of *C. abortus*, 1B. This strain is thought to be temperature sensitive and attenuated due to a lack of growth at temperatures within a host (39.5º C, the body temperature of sheep) (Buendia et al. 2009; Longbottom et al. 2018). A recent genomic study by Longbottom et al. (2018) failed to find a genetic basis for temperature sensitivity of

*C. abortus* strain 1B and previous studies have discussed inactivating *C. abortus* attenuated vaccines to reduce the risk of chlamydial related ectopic pregnancies (Buendia et al. 2009; Caro et al. 2005). In the future, the current *C. abortus* vaccine can be optimized by estimating the effect size of chlamydial load, host immune response, and disease prevalence for new *C. abortus* immunizations and comparing these to the 1B effect sizes. Thus, vaccine optimization in this way may be useful for evaluating the efficacy of new immunizations to commercially available vaccines (e.g. hepatitis (A and B), influenza, and human papillomavirus).

**2.6 Conclusion**

Our meta-analysis shows that on average, most chlamydial immunizations against either *C. trachomatis* or *C. muridarum* were effective at reducing host chlamydial load, protecting vaccinated individuals against future chlamydial infection. Additionally, many chlamydial immunization types increased host interferon gamma (IFNg), and anti-*Chlamydia* antibodies immunoglobulin A (IgA), IgG1, and IgG2a. Our analysis of all studies indicates that IgA and IgG2a were correlated with decreases in both *C. muridarum* and *C. trachomatis* chlamydial load. When one study was removed, we found a relationship between the average change in chlamydial load and the average change in IFNg for immunizations against *C. trachomatis*. A relationship between average chlamydial load decrease and IFNg increase for immunizations against *C. muridarum* was less obvious, likely due to the difficulties in measuring IFNg *ex vivo*. We identified the most promising chlamydial immunizations consisting of single proteins, viral and bacterial vectors expressing chlamydial antigenic proteins, dendritic cell adoptive transfer, and dead whole pathogen, however vaccinologists should also consider immunization route, protection against multiple chlamydial serovars,

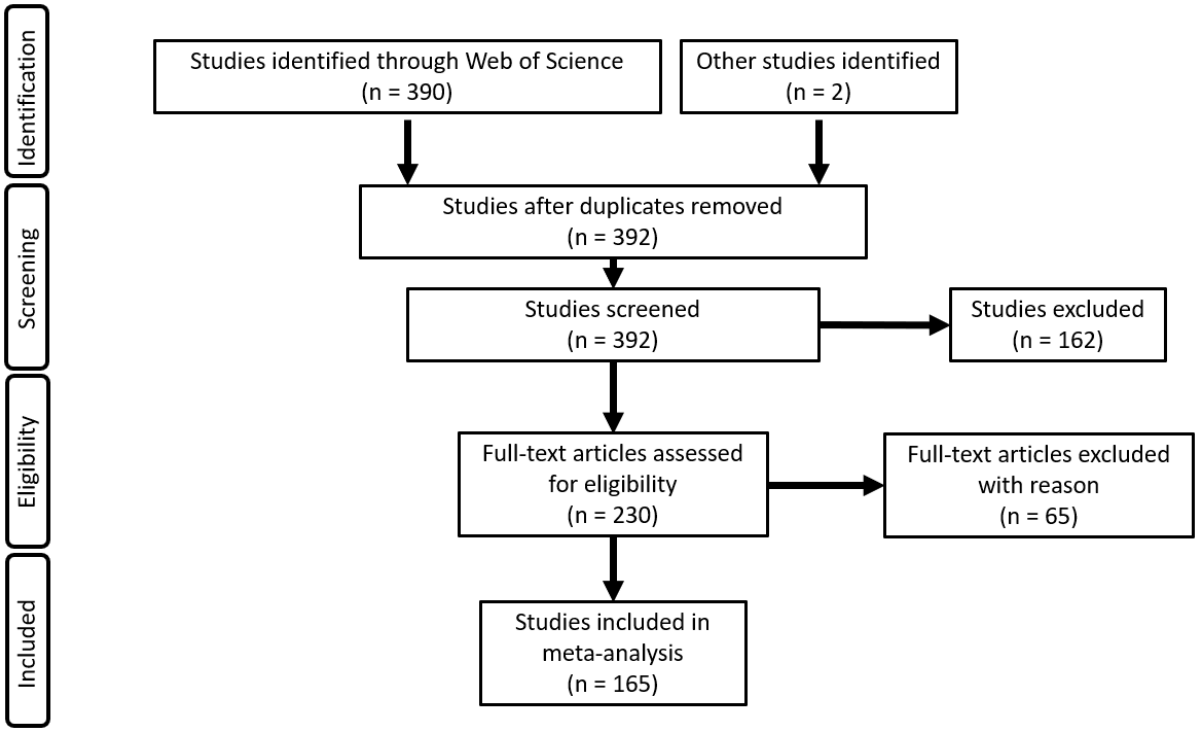
disease potential, and immunization creation costs in future chlamydial immunization trials. Our study describes methods that could be applied to analyse vaccination studies against other pathogens where a large quantity of literature might exist such as *Neisseria gonorrhoeae* and human immunodeficiency virus.

**2.7 Key Issues**

1. The number of vaccine development studies has increased in recent years resulting in large quantities of experimental results, making it difficult to identify the most efficacious vaccine candidates.
2. We undertook a systematic review of vaccine candidates against *Chlamydia*, a field where a vaccine for humans does not yet exist.
3. Our analysis shows the most effective vaccine candidates (immunizations) that reduce host chlamydial load are single recombinant protein, viral and bacterial vectors, dendritic cell adoptive transfer, and dead whole pathogen immunizations.
4. Additionally, most immunizations increased key anti-*Chlamydia* host immune parameters (IFNg, IgA, IgG1, IgG2a).
5. We found a correlation between the average IgA or IgG2a increase and average chlamydial load decrease after immunization against *C. muridarum* or *C. trachomatis*.
6. Across all studies, we found a relationship between average chlamydial load decrease and average IFNg increase for immunizations against *C. trachomatis*, but not for immunizations against *C. muridarum*; likely complicated by the difficulty in measuring IFNg.

7. The chlamydial vaccine development field is highly bias toward studies showing chlamydial load reductions and immune parameter increases, limiting lessons learned from unpublished studies and experiments where negative results were obtained.
8. This study shows which vaccine candidates most effectively reduced chlamydial load, however, other factors such as disease prevention, delivery route, adjuvant usage, and protection against multiple chlamydial serovars should be considered in future studies.
9. The methods used in this study can be applied to identify effective vaccine candidates against other pathogens where a vaccine does not currently exist (e.g. *Neisseria gonorrhoeae* and HIV).

**Chapter 2 Supplementary Material**




**Supplementary Figure 2.1.** Studies identified through Web of Science and those included in our meta-analysis filtered according to the PRISMA guidelines described by Moher et al. (2009).

**Supplementary Table 2.1.** All 230 studies identified in our systematic search of the literature that were assessed using the full-text articles for eligibility in our meta-analysis. Paper ID, Primary author, and year are all reported in extracted data within Supplementary dataset 2.1. Additionally, whether the study was used in the meta-analysis (for exclusion justification see Supplementary dataset 2.1, tab 3) and the range of effect sizes estimated (labelled in Supplementary dataset 2.1 by Unique ID). For the title of each paper, please refer to Supplementary dataset 2.1, tab 2).

| Paper ID | Primary author | Year | Data extracted? | Unique ID in dataset |
|---|---|---|---|---|
| 1 | Alvarez | 2015 | Yes | 1-9 |
| 2 | Andrew | 2011 | Yes | 11-14 |
| 3 | Andrew | 2013 | No | -- |
| 4 | Ausiello | 2005 | No | -- |
| 5 | Badamchi-Zadeh | 2015 | Yes | 15-40 |
| 6 | Badamchi-Zadeh | 2016 | Yes | 41-105 |
| 7 | Bandholtz | 2002 | Yes | 106-123 |
| 8 | Batteiger | 1993 | Yes | 124-134 |
| 9 | Berry | 2004 | No | -- |
| 10 | Biesenkamp-Uhe | 2007 | Yes | 135-138 |
| 11 | Boje | 2016 | Yes | 139-142 |
| 12 | Bommana | 2017 | No | -- |
| 13 | Brown | 2012 | Yes | 177-226 |
| 14 | Brunham | 1999 | Yes | 143-153 |
| 15 | Brunner | 2006 | Yes | 154-159 |
| 16 | Buendia | 2009 | Yes | 160-168 |
| 17 | Buendia | 1999 | No | -- |
| 18 | Bulir | 2016 | Yes | 169-176 |
| 19 | Buxton | 1981 | No | -- |
| 20 | Cambridge | 2013 | No | -- |
| 21 | Campos | 1995 | Yes | 227-233 |
| 22 | Carey | 2011 | Yes | 261-284 |
| 23 | Carey | 2010 | No | -- |
| 24 | Carmichael | 2011 | Yes | 234-236 |
| 25 | Caro | 2005 | Yes | 237-242 |
| 26 | Caro | 2001 | Yes | 243-260 |
| 27 | Chaganty | 2010 | Yes | 285-302 |
| 28 | Chalmers | 1997 | No | -- |
| 29 | Chang | 1964 | No | -- |
| 30 | Cheng | 2009 | Yes | 303-304 |
| 31 | Cheng(a) | 2011 | Yes | 340-347 |
| 32 | Cheng(b) | 2011 | Yes | 305-319 |
| 33 | Cheng(c) | 2011 | Yes | 348-364 |
| 34 | Cheng | 2014 | Yes | 320-339 |

| | | | | |
|---|---|---|---|---|
| 35 | Childs | 2012 | No | -- |
| 36 | Collier | 1966 | No | -- |
| 37 | Collier | 1967 | No | -- |
| 38 | Cong | 2007 | Yes | 365-432 |
| 39 | Conlan | 1990 | No | -- |
| 40 | Cunningham | 2009 | Yes | 489-520 |
| 41 | Desclozeaux (a) | 2017 | No | -- |
| 42 | Desclozeaux (b) | 2017 | Yes | 1002-1018 |
| 43 | Dixit | 2018 | No | -- |
| 44 | Dixit | 2014 | Yes | 433-460 |
| 45 | Donati | 2003 | No | -- |
| 46 | Eko (a) | 2011 | Yes | 521-549 |
| 47 | Eko | 2004 | Yes | 461-488 |
| 48 | Eko (b) | 2011 | Yes | 550-615 |
| 49 | Ekong | 2009 | Yes | 616-709 |
| 50 | Fairley | 2013 | Yes | 710-721 |
| 51 | Faludi | 2009 | Yes | 770-775 |
| 52 | Faludi | 2011 | Yes | 805-816 |
| 53 | Farris | 2010 | Yes | 722-733 |
| 54 | Finco | 2011 | Yes | 734-739 |
| 55 | Ganda | 2017 | Yes | 740-769 |
| 56 | Giadinis | 2000 | No | -- |
| 57 | Grayston | 1962 | No | -- |
| 58 | Grayston | 1963 | No | -- |
| 59 | Guiot | 2008 | Yes | 776-783 |
| 60 | Gupta | 2016 | No | -- |
| 61 | Hadad | 2016 | Yes | 784-801 |
| 62 | Harkinezhad | 2009 | Yes | 802 |
| 63 | Harley | 2010 | No | -- |
| 64 | Hayes | 1991 | Yes | 803-804 |
| 65 | He (a) | 2007 | No | -- |
| 66 | He (b) | 2007 | Yes | 817-822 |
| 67 | He | 2017 | Yes | 823 |
| 68 | Hechard | 2002 | Yes | 824-829 |
| 69 | Hechard (a) | 2003 | Yes | 830-842 |
| 70 | Hechard (b) | 2003 | Yes | 843-848 |
| 71 | Hechard | 2004 | Yes | 849-862 |
| 72 | Hickey | 2009 | Yes | 863-910 |
| 73 | Hickey | 2010 | Yes | 1019-1045 |
| 74 | Igietseme | 2000 | Yes | 911-946 |
| 75 | Inic-Kanada | 2016 | Yes | 947-972 |
| 76 | Inic-Kanada | 2015 | Yes | 973-982 |
| 77 | Ishizaki | 1992 | No | -- |
| 78 | Jiang (a) | 2017 | Yes | 983-1001 |
| 79 | Jiang (b) | 2017 | Yes | 1046-1153 |
| 80 | Jiang | 2008 | Yes | 1154-1163 |

| | | | | |
|---|---|---|---|---|
| 81 | Johnson | 2012 | No | -- |
| 82 | Jones | 1995 | No | -- |
| 83 | Kari | 2009 | Yes | 1164-1220 |
| 84 | Kari | 2011 | Yes | 1221-1234 |
| 85 | Karunakaran | 2008 | No | -- |
| 86 | Karunakaran | 2015 | Yes | 1235-1237 |
| 87 | Kawa | 2004 | No | -- |
| 88 | Keisler | 1989 | No | -- |
| 89 | Khan (a) | 2016 | Yes | 1238-1261 |
| 90 | Khan (b) | 2016 | Yes | 1262-1273 |
| 91 | Khan | 2014 | Yes | 1274-1345 |
| 92 | Knight | 1995 | Yes | 1346-1351 |
| 93 | Knitz | 2003 | No | -- |
| 94 | Kollipara | 2012 | Yes | 1352-1413 |
| 95 | Kollipara (a) | 2013 | No | -- |
| 96 | Kollipara (b) | 2013 | Yes | 1414-1533 |
| 97 | Koroleva | 2017 | Yes | 1534-1559 |
| 98 | Kuczkowska | 2017 | Yes | 1560-1586 |
| 99 | Li (a) | 2008 | Yes | 1587-1614 |
| 100 | Li | 2007 | Yes | 1615-1695 |
| 101 | Li (a) | 2010 | Yes | 1696-1746 |
| 102 | Li (b) | 2008 | Yes | 1747-1780 |
| 103 | Li | 2013 | No | -- |
| 104 | Li (b) | 2010 | No | -- |
| 105 | Li | 2012 | Yes | 1781-1814 |
| 106 | Li (c) | 2008 | Yes | 1860-1889 |
| 107 | Liang | 2016 | Yes | 1815-1823 |
| 108 | Ling (a) | 2011 | Yes | 1824-1859 |
| 109 | Ling (b) | 2011 | Yes | 2110-2133 |
| 110 | Liu | 2012 | No | -- |
| 111 | Liu | 2015 | Yes | 1890-1899 |
| 112 | Loots | 2006 | Yes | 1900-1935 |
| 113 | Lorenzen | 2015 | Yes | 1936-2025 |
| 114 | Lu | 2013 | Yes | 2026-2056 |
| 115 | Lu | 2012 | Yes | 2134-2172 |
| 116 | Lu | 2010 | Yes | 2057-2066 |
| 117 | Lu | 2002 | Yes | 2067-2109 |
| 118 | MacDonald | 1984 | Yes | 2173-2178 |
| 119 | Macmillan | 2007 | Yes | 2179-2202 |
| 120 | Masubuchi | 2010 | No | -- |
| 121 | McKercher | 1973 | No | -- |
| 122 | McNeilly | 2007 | Yes | 2203-2244 |
| 123 | Motin | 1999 | No | -- |
| 124 | Murdin | 1993 | No | -- |
| 125 | Murphey | 2006 | Yes | 2307-2331 |
| 126 | Murthy | 2007 | Yes | 2245-2306 |

| | | | | |
|---|---|---|---|---|
| 127 | Murthy | 2006 | Yes | 2332-2346 |
| 128 | Neumann | 1997 | No | -- |
| 129 | Oconnell | 2007 | No | -- |
| 130 | Olivares-Zavaleta | 2010 | Yes | 2679-2703 |
| 131 | Olivares-Zavaleta | 2014 | Yes | 2569-2586 |
| 132 | Olsen | 2014 | Yes | 2379-2386 |
| 133 | Olsen | 2015 | Yes | 2347-2359; 4330-4332 |
| 134 | Olsen | 2017 | Yes | 2387-2416 |
| 135 | Olsen | 2010 | Yes | 2360-2378 |
| 136 | Omeara | 2017 | Yes | 2417-2568 |
| 137 | Omeara | 2014 | Yes | 2599-2625 |
| 138 | Omeara | 2013 | No | -- |
| 139 | Omeara | 2016 | Yes | 2626-2678 |
| 140 | Ou | 2013 | Yes | 2587-2598 |
| 141 | Pais | 2017 | Yes | 2704-2722 |
| 142 | Pal (a) | 1999 | No | -- |
| 143 | Pal | 2002 | No | -- |
| 144 | Pal | 2017 | Yes | 2723-2762 |
| 145 | Pal (a) | 2003 | Yes | 2795-2811 |
| 146 | Pal (a) | 2005 | No | -- |
| 147 | Pal (b) | 2003 | No | -- |
| 148 | Pal (b) | 2005 | Yes | 2763-2770 |
| 149 | Pal | 2006 | Yes | 2771-2776 |
| 150 | Pal (b) | 1999 | Yes | 2812-2819 |
| 151 | Pal | 2010 | No | -- |
| 152 | Pal | 2015 | Yes | 2777-2794 |
| 153 | Pal | 2001 | Yes | 2820-2835 |
| 154 | Pal | 1997 | Yes | 2836-2857 |
| 155 | Pan | 2015 | Yes | 2858-2894 |
| 156 | Penttila | 2004 | Yes | 2977-2981 |
| 157 | Penttila | 2000 | Yes | 2982-3007 |
| 158 | Peterson | 1996 | No | -- |
| 159 | Peterson | 1999 | Yes | 3008 |
| 160 | Pinchuk | 2005 | Yes | 2895-2906 |
| 161 | Popov | 1985 | No | -- |
| 162 | Qu | 2015 | No | -- |
| 163 | Ralli-Jain | 2010 | Yes | 3009-3027 |
| 164 | Ran | 2017 | Yes | 2907-2916 |
| 165 | Rey-Ladino | 2005 | Yes | 2917-2919 |
| 166 | Rodolakis | 1979 | No | -- |
| 167 | Rose | 2018 | No | -- |
| 168 | Sampaio | 1963 | No | -- |
| 169 | Schautteet | 2012 | Yes | 3177-3277 |
| 170 | Schautteet (a) | 2011 | Yes | 2920-2976 |

| | | | | |
|---|---|---|---|---|
| 171 | Schautteet (b) | 2011 | Yes | 3028-3031 |
| 172 | Schmeer | 1987 | No | -- |
| 173 | Shaw | 2002 | Yes | 3032-3033 |
| 174 | Shaw | 2001 | Yes | 3034-3041 |
| 175 | Shewen | 1980 | No | -- |
| 176 | Singh | 2006 | Yes | 3042-3101 |
| 177 | Skwor | 2010 | No | -- |
| 178 | Sobinoff | 2015 | Yes | 3102-3111 |
| 179 | Stary | 2015 | Yes | 3112-3159 |
| 180 | Su | 1992 | No | -- |
| 181 | Su | 1993 | No | -- |
| 182 | Su | 1998 | Yes | 3160-3170 |
| 183 | Su | 1995 | Yes | 3308-3342 |
| 184 | Sun | 2009 | Yes | 3171-3176 |
| 185 | Svanholm | 2000 | Yes | 3278-3293 |
| 186 | Tan | 1990 | No | -- |
| 187 | Thorpe | 2007 | Yes | 3294-3307 |
| 188 | Tifrea | 2014 | Yes | 3343-3370 |
| 189 | Tifrea (a) | 2013 | Yes | 3371-3388 |
| 190 | Tifrea (b) | 2013 | Yes | 3389-3404 |
| 191 | Tifrea | 2011 | Yes | 3596-3611 |
| 192 | Tu | 2014 | Yes | 3405-3420 |
| 193 | Van Loock | 2004 | Yes | 3612-3621 |
| 194 | Vanrompay | 2001 | Yes | 3421-3440 |
| 195 | Vanrompay (a) | 1999 | Yes | 3622-3663 |
| 196 | Vanrompay (b) | 1999 | Yes | 3441-3454 |
| 197 | Verminnen | 2010 | Yes | 3455-3514 |
| 198 | Verminnen | 2005 | Yes | 3515-3519 |
| 199 | Visan | 2016 | Yes | 3520-3559 |
| 200 | Wang | 2009 | Yes | 3560-3595 |
| 201 | Wang | 2017 | Yes | 3712-3757 |
| 202 | Wang | 1988 | No | -- |
| 203 | Wasmoen | 1992 | No | -- |
| 204 | Waugh | 2016 | Yes | 3664-3711 |
| 205 | Waugh | 2015 | Yes | 3707 |
| 206 | Wen | 2016 | Yes | 3758-3761 |
| 207 | Wern | 2017 | Yes | 3762-3801 |
| 208 | Westbay | 1994 | No | -- |
| 209 | WhittumHudson | 1996 | Yes | 3842-3856 |
| 210 | Wilsmore (a) | 1990 | No | -- |
| 211 | Wilsmore (b) | 1990 | No | -- |
| 212 | Xu | 2011 | Yes | 4036-4061 |
| 213 | Yu | 2009 | Yes | 3802-3841 |
| 214 | Yu | 2010 | Yes | 3857-3903 |
| 215 | Yu | 2014 | Yes | 3904-3951 |
| 216 | Yu | 2012 | Yes | 3952-4035 |

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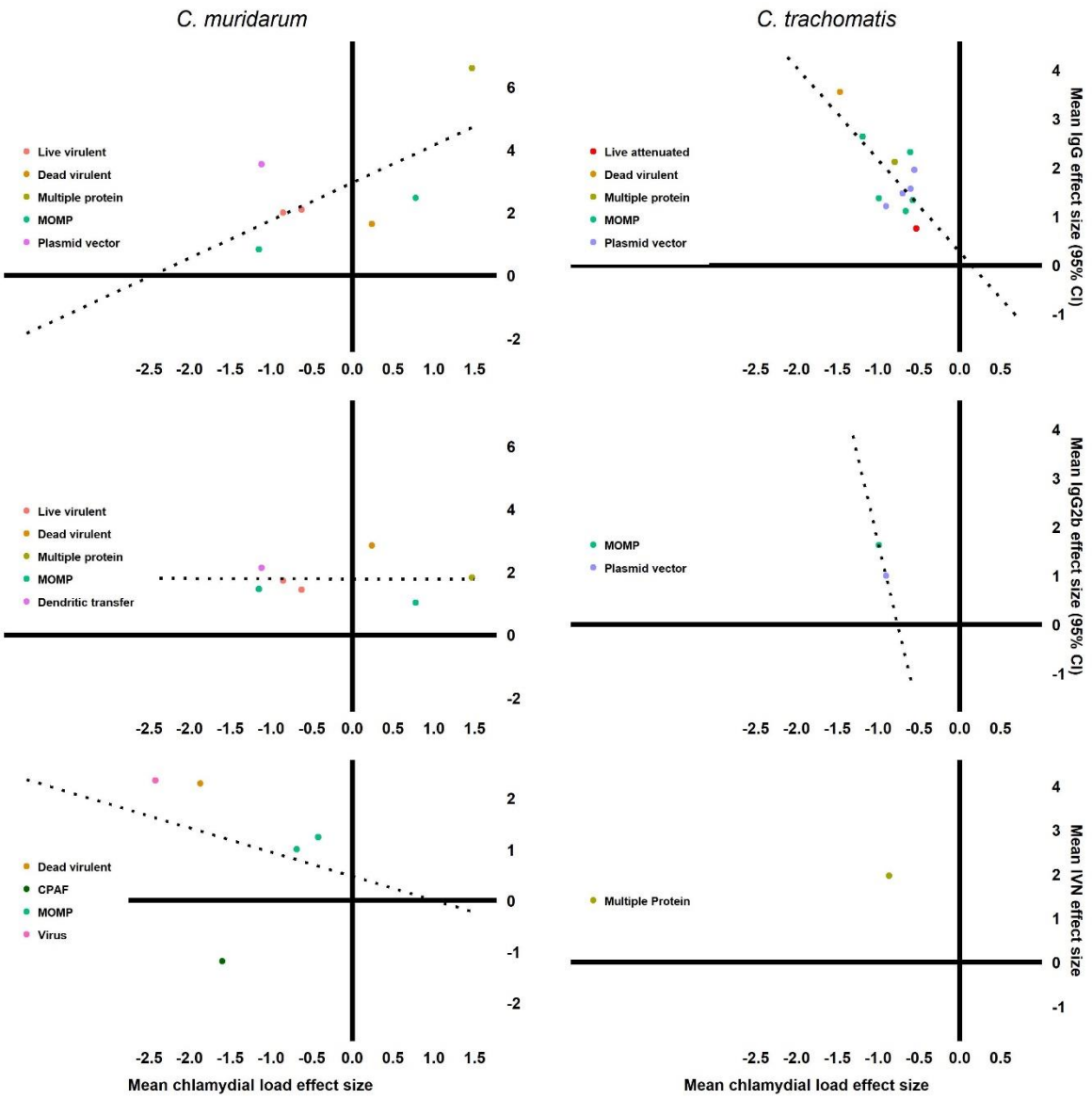
|---|---|---|---|---|
| 217 | Yu | 2011 | Yes | 4062-4121 |
| 218 | Zhang | 1997 | Yes | 4122-4133 |
| 219 | Zhang (a) | 1999 | Yes | 4134-4147 |
| 220 | Zhang (b) | 1999 | Yes | 4148-4157 |
| 221 | Zhang | 2000 | Yes | 4158-4175 |
| 222 | Zhang | 2009 | Yes | 4176-4195 |
| 223 | Zhang | 2013 | Yes | 4196-4231 |
| 224 | Zheng | 2007 | Yes | 4232-4249 |
| 225 | Zhou | 2007 | Yes | 4250-4254 |
| 226 | Zhu | 2010 | No | -- |
| 227 | Zhu | 2014 | Yes | 4255-4329 |
| 228 | Coler | 2009 | Yes | 4333-4363 |
| 229 | Knudsen | 2016 | Yes | 4364-4453 |
| 230 | Cotter | 1995 | No | -- |

**Supplementary Table 2.2.** Chlamydial immunization immune categories, effect sizes, the number of studies, and the host immune parameters included for each category from the complete dataset.

| Immune category | Effect sizes | Studies | Immune parameters included in category |
|---|---|---|---|
| Chlamydial load (C.L.) | 1,424 | 110 | Chlamydial load |
| Interferon gamma (IFNg) | 348 | 71 | IFNg |
| Immunoglobulin A (IgA) | 327 | 45 | IgA |
| IgG | 684 | 55 | IgG |
| IgG1 | 119 | 32 | IgG1 |
| IgG2a | 155 | 36 | IgG2a |
| IgG2b | 51 | 12 | IgG2b |
| IgG2c | 8 | 2 | IgG2c |
| IgG3 | 5 | 4 | IgG3 |
| In vitro neutralization (IVN) | 103 | 16 | In vitro neutralization |
| Th1 cytokine (Th1 cyt.) | 97 | 22 | IL2, IL12, TNFa |
| Th17 cytokine (Th17 cyt.) | 63 | 14 | IL17, IL17a |
| Th2 cytokine (Th2 cyt.) | 189 | 41 | IL4, IL5, IL10, IL13 |
| Other | 880 | 105 | e.g. Antibody measurement (207), IFNg secreting cells (74), IgM (59), Lymphocyte proliferation (75), T cell proliferation (100) |

**Supplementary Table 2.3.** Host type, effect size number, and total number of studies from the full dataset.

| Host | Effect sizes | Studies |
|---|---|---|
| Mice | 3,465 | 129 |
| Koalas | 355 | 8 |
| Pigs | 268 | 6 |
| Birds | 203 | 10 |
| Non-human primates | 119 | 6 |
| Guinea pigs | 15 | 2 |
| Cats | 14 | 2 |
| Sheep | 10 | 1 |
| Cows | 4 | 1 |




**Supplementary Figure 2.2.** Host immunoglobulin G (IgG), IgG2b, and in vitro neutralization (IVN) plotted against chlamydial load for immunizations against *C. muridarum* and *C. trachomatis*. Dashed lines indicate a trendline created from a linear regression. To better visualize the data, all values (mean values and confidence intervals, CIs) were transformed using the cube root function. For Spearman's ranked correlation results see Table 2.2.

**Supplementary Table 2.4.** Estimate (i.e. average) of change in chlamydial load from the meta-regression, variation (lower and upper confidence intervals), and sample size for chlamydial immunizations against *C. trachomatis* in pigs, *C. trachomatis* in non-human primates, *C. abortus* in mice, *C. pneumoniae* in mice, and *C. pecorum* in koalas.

| Species (host) | Immunization | | Estimate (2.5%, 97.5% CI) | Effect sizes | Studies |
|---|---|---|---|---|---|
| *C. trachomatis* (pigs) | Whole pathogen | Dead virulent | -1.978 (-3.080, -0.876) | 2 | 1 |
| | Multiple recombinant proteins | | -0.836 (-1.691, 0.020) | 5 | 2 |
| *C. trachomatis* (non-human primates) | Whole pathogen | Live attenuated | -0.615 (-0.835, -0.396) | 30 | 2 |
| | | Dead virulent | 0.752 (-0.439, 1.943) | 6 | 1 |
| | Single recombinant proteins | MOMP | -0.200 (-0.492, 0.092) | 21 | 1 |
| *C. abortus* (mice) | Whole pathogen | Live attenuated | -0.470 (-0.752, -0.189) | 18 | 8 |
| | | Dead attenuated | -1.169 (-1.691, -0.647) | 7 | 3 |
| | | Dead virulent | -1.423 (-2.241, -0.604) | 4 | 1 |
| | Single recombinant proteins | MOMP | $-6.984\ e^{-6}$ (-0.880, 0.880) | 1 | 1 |
| | Plasmid expression vector | | -0.046 (-0.256, 0.163) | 23 | 8 |
| | Viral vector | | -0.003 (-0.843, 0.836) | 1 | 1 |
| *C. pneumoniae* (mice) | Single recombinant proteins | MOMP | 0.109 (-0.567, 0.785) | 1 | 1 |
| | Plasmid expression vector | | -0.651 (-0.894, -0.408) | 18 | 4 |
| | Viral vector | | -0.649 (-1.200, -0.097) | 3 | 1 |
| *C. pecorum* (koalas) | Multiple recombinant proteins | | -0.413 (-0.699, -0.127) | 4 | 2 |
| | Single recombinant proteins | Pmp | 0.359 (-0.064, 0.783) | 2 | 1 |

**Supplementary Table 2.5.** Pathogen, number of studies, and Boolean operators used in a Web of Science search.

| Pathogen | Number of articles | Boolean operators |
|---|---|---|
| Herpes simplex virus type 1 | 110 | **TITLE:** (herpes simplex virus type 1 AND (Vaccin* OR Immun*)) *AND* **TOPIC:** ((Vaccin*)) |
| *Trichomonas vaginalis* | 17 | **TITLE:** (trichomon* AND (Vaccin* OR Immun*)) *AND* **TOPIC:** ((Vaccin*)) |
| *Treponema pallidum* | 50 | **TITLE:**(treponema* AND (Vaccin* OR Immun*)) *AND* **TOPIC:** ((Vaccin*)) *OR* **TITLE:** (syphilis* AND (Vaccin* OR Immun*)) *AND* **TOPIC:** ((Vaccin*)) |
| *Neisseria gonorrhoeae* | 47 | **TITLE:** (gonorrh* AND (Vaccin* OR Immun*)) *AND***TOPIC:** ((Vaccin*)) |
| Human immunodeficiency virus | 1,638 | **TITLE:** (human immunodeficiency virus AND (Vaccin*)) *AND* **TOPIC:** ((Vaccin*)) *OR* **TITLE:** (HIV-1* AND (Vaccin*)) *AND* **TOPIC:** ((Vaccin*)) |

**Supplementary text 2.1**

*C. trachomatis immunizations in non-mouse models*

The small number of studies published using non-mouse hosts make the identification of efficacious chlamydial immunizations and the identification of trends difficult. Here we discuss the results from non-mouse studies using a finer scope than that used to analyse studies using the mouse model. In the following sections we will compare results from the available data (and in some cases the results from individual studies) to the trends we identified previously using data obtained from chlamydial immunization experiments using mouse model.

*Pigs.* Two studies investigated the effects of *C. trachomatis* immunizations on chlamydial load in Gottingen minipigs (Boje et al. 2016; Lorenzen et al. 2015). Pigs were used to test the effects of chlamydial immunizations as they were more closely related to humans compared to mice and had much larger genital tracts for sampling as compared to the mouse model (Schautteet et al. 2012). Between the two studies, we estimated seven effect sizes for two immunization types consisting of either multiple recombinant proteins or dead whole chlamydial pathogen (see Supplementary Table 2.4). Both immunization types were delivered with the adjuvant CAF01. On average, both immunizations types had a negative effect on chlamydial load. Similar to *C. muridarum* studies, dead whole chlamydial pathogens elicit protection when delivered with an immunogenic chemical adjuvant. All of the multiple protein immunizations used in pigs consisted of the same two proteins: Hirep 1 (consisting of B-cell epitopes for three serovars of MOMP) and CTH93 (consisting of T-cell epitopes, CTH043, CTH414, and one serovar of MOMP). The use of MOMP in these multiple protein immunizations further highlights the importance of this immunogenic protein as it

has been shown to be an effective protein on its own (see results from experiments with *C. trachomatis* immunizations in mice).

*Non-human primates.* In our meta-analysis, four studies investigated the effects of immunizations on non-human primates against *Chlamydia trachomatis* (Kari et al. 2009; Kari et al. 2011; Macdonald et al. 1984; Olivares-Zavaleta et al. 2014). Arguably the closest related host species to humans used in chlamydial immunization studies, the two species of non-human primates used were *Aotus trivirgatus* and *Macaca fascicularis*. Three immunization types were used: dead virulent whole chlamydial pathogen, live attenuated whole chlamydial pathogen, or the single recombinant protein, MOMP (see Supplementary Table 2.4). Irradiated virulent *C. trachomatis* E.B.s and R.B.s (i.e. dead virulent whole pathogen) without an adjuvant had no effect on chlamydial load. The live attenuated *C. trachomatis* strain A2497P- was used without an adjuvant that resulted in a decrease in chlamydial load. The single protein immunization consisting of MOMP with the adjuvants CpG and Montanide or both Freund's Complete and Incomplete Adjuvant reduced chlamydial load.

*C. abortus immunizations in mice.* Multiple hosts including humans can be infected with *C. abortus*, though bovine and ovine infections are more common (Buendia et al. 2009; Caro et al. 2005; Caro et al. 2001). As the name suggests, fetal death is one symptom of *C. abortus* infection and is of financial concern for farmers raising livestock. The use of bioinformatic analyses have identified genes responsible for the attenuation for strains in the *C. abortus* 1B vaccine (Burall et al. 2009). Genomic analyses could play a critical role in identifying genes that cause attenuated virulence, and the possibility of creating an attenuated *C. trachomatis* vaccine resulting from these analyses seems entirely possible.

*C. pneumoniae immunizations in mice.* Unlike the chlamydial species discussed in this study, *C. pneumoniae* typically infects respiratory mucosal linings and can lead to pneumonia in humans. Immunizations that boost the immune response of respiratory sites against this chlamydial species may be beneficial for other respiratory pathogens such as *C. psittaci*. Six studies tested the effects of chlamydial immunizations in mice against *C. pneumoniae* using three immunizations: single recombinant protein (MOMP), plasmid expression vector, and viral vector. From these studies, plasmid expression vector immunizations were most effective at reducing chlamydial load. These immunizations consisted of plasmids expressing at least one of the following: MOMP, heat shock protein 60, or outer membrane protein 2.

*C. pecorum immunizations in koalas.* The only extant member of Phascolarctidae, *Phascolarctos cinereus* (commonly called the koala), is a marsupial host used in eight chlamydial immunization studies against the chlamydial species, *Chlamydia pecorum*. Some koala populations experience high rates of chlamydial infection (<80%; Polkinghorne 2013) that contribute to the reduction of koala populations and localized extinctions (Rhodes et al. 2011). Developing a koala specific chlamydial immunization is critical to the survival of individuals in these diseased populations. Thus, some studies have measured the effects of chlamydial immunizations using wild individuals that were monitored over several months after immunization. The chlamydial immunizations were delivered either as a single protein or as multiple proteins which, in some cases, contain a similar protein from different chlamydial serovars (e.g. MOMP from serovar A, F, and G; see Supplementary Figure 2.6). Immunizations that elicit strong immune responses against multiple serovars are ideal as some individuals are infected with different chlamydial serovars (Marsh et al. 2011). On average the multiple protein (MOMP A, F, G) immunization was more effective than the

peripheral membrane protein (Pmp) immunization. These effect sizes were estimated based on data collected from wild animals with an unknown history of infection. Thus, the two effect sizes in Supplementary Table 2.4 represent a combined therapeutic and protective effect as some individuals were infected at the time of vaccination (timepoint 0).

## Chapter 3: Capturing complex vaccine-immune-disease relationships for free-ranging koalas: higher chlamydial loads are associated with less IL17 expression and more chlamydial disease

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Author's contributions: DL, PT, BQ, and SC conceived the study design. PT and JH collected the data. DL performed the analysis. All authors contributed to interpreting the results and drafting of the manuscript.

**3.1 Abstract**

**Background:** Chlamydial disease is a major factor negatively affecting koala populations. Vaccination is a promising management option that would result in immune-mediated protection against disease. Measuring and assessing vaccine efficacy can be challenging owing to both direct and indirect interactions caused by vaccination. In this study, we investigate vaccine-immune-chlamydial load-disease relationships from MOMP (major outer membrane protein) vaccine trials to protect healthy free-ranging koalas against *Chlamydia*-related diseases.

**Methods:** We created *a priori* hypotheses and tested these hypotheses using previously published data collected by Waugh et al. (2016) and Desclozeaux et al. (2017). We created these hypotheses to perceive direct and indirect interactions from koalas vaccinated six months prior. As previous studies have found no effect of vaccination on disease, we sought to test indirect links of vaccination to disease. Each hypothesis was tested as a structural equation model separately for either the urogenital or the ocular site to evaluate possible causality among measured variables. Model averaging was used as multiple models fit the data, and the strength of relationships was examined through averaged coefficients and the raw data.

**Results:** We found more relationships in urogenital models as compared to ocular models, particularly those with interleukin 17 (IL17) mRNA expression compared to models with interferon gamma (IFNγ) expression. In the averaged model with IL17, urogenital chlamydial load was positively associated with disease and negatively associated with IL17 expression. MOMP vaccination had a trending effect for reducing urogenital chlamydial load and also had a strong effect on increasing IL17 expression. Not surprisingly, urogenital chlamydial

load was a positive predictor for the development of urogenital disease at six months post-vaccination. The averaged model suggests that IL17 expression was not linked to disease at the ocular or urogenital sites.

**Conclusions:** Despite multiple potential sources of variation owing to the koalas in this study being free-ranging, our analyses provide unique insights into the effects of vaccinating against *Chlamydia*. Using structural equation modelling, this study has helped illuminate that the expression of the immune cytokine IL17 is linked to MOMP vaccination, and animals with a high urogenital chlamydial load expressed less IL17 and were more likely to develop disease, enhancing previous investigations. Going beyond univariate statistics, the methods used in this study can be applied to other preclinical vaccination experiments to identify important direct and indirect factors underpinning the effects of a vaccine.

Keywords: structural equation model, Chlamydia, vaccine, koala, cytokine

## 3.2 Introduction

Koala (*Phascolarctos cinereus*) populations, particularly in the Australian Capital Territory and in the Australian states of New South Wales and Queensland, have suffered staggering losses over recent years (Adams-Hosking et al. 2016; McAlpine et al. 2015; Melzer et al. 2000; Rhodes et al. 2015) leading to their conservation status being listed as 'vulnerable' per the IUCN (Woinarski et al. 2015). A number of factors negatively affect koala populations including: habitat loss (Mcalpine et al. 2006; Melzer et al. 2000), climate change(Seabrook et al. 2011), bushfires (Lunney et al. 2007), motor vehicle accidents (Mcalpine et al. 2006), dog attacks (Lunney et al. 2007), and disease (Rhodes et al. 2011). Deterministic age structured matrix models (Rhodes et al. 2011) of four of these factors (habitat loss, dog attacks, motor vehicle collisions, and disease) indicate that reducing the prevalence of disease may stabilize koala populations. The magnitude of disease-related mortality within a given population is potentially exacerbated by environmental stressors including climate change, habitat loss resulting from urbanisation, and environmental disasters such as bushfires, though to our knowledge no study has investigated this in wild koala populations (McCallum et al. 2018). Amid its variable magnitude, the reduction of disease has been a key management strategy of this species (Beyer et al. 2018; McAlpine et al. 2015; Polkinghorne et al. 2013; Rhodes et al. 2011).

*Chlamydia* is an obligate, intracellular bacterium that is the most prevalent disease-causing pathogen in wild koalas (Jackson et al. 1999; Polkinghorne et al. 2013). Koalas are hosts to two bacterial species of *Chlamydia* (*C. pecorum* and *C. pneumoniae*), however, modern vaccine candidates target the more prevalent species, *C. pecorum*, that typically infects epithelial cells in the ocular and urogenital mucosa (Jackson et al. 1997). Sterility and

disease-related mortality as a result of chlamydial infections have a direct, negative impact on koala population dynamics and are relatively common among some free-ranging koala populations (Beyer et al. 2018; Loader 2010). Chlamydial infections in koalas are treatable with antibiotics, but this management strategy is potentially fatal for the specialized microflora in the koala gastrointestinal tract that is necessary for eucalyptus digestion (Robbins et al. 2018). Vaccinating koalas is a promising tool for disease management that is modelled to have clear benefits, particularly at the population scale. Modelling by Craig et al. (Craig et al. 2014) suggested that chlamydial vaccination could stabilize koala populations after five years of using a vaccine (with protective efficacy of 75%) administered to around 10% of koalas per year. Such a vaccine does not yet exist, however, many studies within the last decade have advanced the development of a vaccine for koalas against *Chlamydia*.

Much of the foundational work for chlamydial vaccine development has used captive koalas under controlled veterinary conditions (reviewed by Phillips et al. 2019). However, field trials that encompass a greater range of natural variables provide a more accurate picture of vaccine efficacy. To date, there are two published field trials testing a chlamydial vaccine using free-ranging koalas. The first study was by Waugh et al. (2016). This vaccine consisted of three chlamydial major outer membrane proteins (MOMP) from three genotypes (A, F, and G) and an Immunostimulating Complex (ISC) adjuvant, that was delivered subcutaneously three times over three months (given at one-month intervals). Chlamydial load and disease were compared six months post-vaccination between vaccinated and control koalas in the 60 koala trial with 54% of individuals being infected with *C. pecorum* at the time of vaccination. Vaccinated animals had increases in anti-*Chlamydia* immunoglobulin G (IgG) and lower levels of chlamydial load and prevalence of disease (at

both the ocular and urogenital sites) compared to control koalas six months after the initial vaccination. The second field study was by Desclozeaux et al. (2017a). This trial tested two vaccine formulations consisting of either MOMP (three genotypes A, F, and G) or PMP (peripheral membrane protein, genotype G). Both vaccines were delivered subcutaneously alongside a tri-adjuvant (PCEP, IDR1002, and polyI:C) and vaccinated groups were compared to non-vaccinated control koalas (21 koalas in each group, 63 in total). Eight koalas (2/21 in the control, and 6/21 in the MOMP vaccine group) were infected with *C. pecorum* at the time of vaccination for a prevalence of 12.7%. Both MOMP and PMP vaccinated koalas had elevated interferon gamma (IFNγ) and interleukin (IL) 17 mRNA expression six months post-vaccination compared to pre-vaccination levels. Additionally, koalas vaccinated with MOMP had lower chlamydial loads compared to control koalas six months post-vaccination. Combined, these field trials have examined enough koalas that, for the first time, more detailed modelling analysis of factors involved in vaccine responses in koalas can be considered.

The koala immune response has been a focal point of research in recent years, particularly to chlamydial infection (reviewed by Madden et al. 2018). Several important cytokines or antibodies in response to chlamydial infection in koalas have been identified: IFNγ, IL17, IL10, tumour necrosis factor alpha (TNFα), IgG, and IgA. As multiple aspects of the koala immune response are poorly understood, researchers often refer to vaccine trials against two other chlamydial species, *C. trachomatis* or *C. muridarum*, where mice are most often used as host models of infection (Lizárraga et al. 2019; Vasilevsky et al. 2014). Of these murine trials, the most commonly measured host cytokine in response to chlamydial infection is IFNγ (Lizárraga et al. 2019), where the expression of IFNγ has been associated

with protection against chlamydial disease (Igietseme et al. 2015). Increases in IFNγ concentration *in vitro* can lead to the degradation of tryptophan, leading to the starvation of *C. trachomatis* of this essential amino acid and inducing chlamydial persistence (an inactive, intracellular pathogen response to external stressors; Beatty et al. 1994). A recent study has shown *C. pecorum* to be resistant to increasing concentrations of IFNγ *in vitro*, suggestive of different immune-evasion mechanisms as compared to *C. trachomatis* (Islam et al. 2018). This difference from *C. trachomatis* (which is sensitive to IFNγ) could lead to a different effect of IFNγ in koalas to *C. pecorum* infection. Lastly, in murine hosts, elevated levels of both IFNγ and IL17 increased the production of inducible Nitric Oxide synthase (iNOS), promoting the production of microbicidal nitric oxide (NO) that correlated with the reduction of chlamydial load (Zhang et al. 2012). Some studies, however, have suggested that an elevated iNOS response to infection (elevated by host cytokines) may be associated with scarring of the fallopian tubes and immunopathogenesis (Agrawal et al. 2011; Refaat et al. 2009). The relationships between chlamydial vaccination, the host immune response (particularly IFNγ and IL17), chlamydial load, and disease is still poorly understood in koalas.

Clearly, a key challenge in chlamydial vaccine research is understanding complex direct and indirect immune-mediated control of infection and disease. In this study, we aimed to model important direct and indirect factors surrounding the vaccination of free-ranging koalas: vaccination status, host immune parameter, chlamydial load, and disease. We used structural equation models to identify the directionality and magnitude of direct and indirect relationships when all four variables are modelled together. This statistical method has been used previously to identify both multiple environmental and individual factors that affect chlamydial disease pathology in koalas (Quigley et al. 2018). We modelled vaccination

status, the expression of an immune parameter (either IFNγ or IL17), chlamydial load, and disease status at two important mucosal sites (ocular and urogenital) using data collected by Waugh et al. (2016) and Desclozeaux et al. (2017a). We tested six hypotheses to 1) identify the relationships between immune parameter and chlamydial load, and 2) identify the relationships between host immune parameter and disease.

## 3.3 Methods

### *3.3.1 Experimental design*

Following vaccination or non-vaccination, control and vaccinated koalas were resampled at an average of 5.95 months (± 0.33, 95% CI, referred to as six months post vaccination hereafter). We adopted a cross-sectional comparison of control to vaccinated free-ranging koalas to determine if our vaccines against *Chlamydia* was effective, given uncontrolled background variation of individual and immune factors. The intent was to assess whether vaccination superseded background individual variation (i.e., immune history, genetics, and seasonality) in parameters, which we know does not protect koalas against *Chlamydia* disease. We note that koalas were sampled across seasons (Spring n=17, Summer n = 3, Autumn n = 9, Winter n = 11), but in preliminary analyses we found no effect of season on measurements of either IL17 (Kruskal Wallis $X^2$ = 1.060, p = 0.787) or IFNγ (Kruskal Wallis $X^2$ = 2.185, p = 0.535).

### *3.3.2 Pooling data from both field trials*

Previously collected data from two field trials of free-ranging koalas vaccinated against *Chlamydia* described by Waugh et al. (2016) and Desclozeaux et al. (2017a) were used in our analysis (see Appendix 3.1 and 3.2 for a summary of the methods for both studies). The two

field trials (different vaccination schedules and different adjuvants) were investigated to determine if differences exist in the data for MOMP vaccinated individuals (PMP vaccinated individuals were excluded). We first used a meta-analysis to compare the effects of vaccination (control versus vaccinated animals) on chlamydial load and disease prevalence at either the ocular or urogenital site for six month post vaccination data between studies (Figure 3.1a). In one trial (Waugh et al. 2016), all koalas (vaccinated and control; see Figure 3.1a) remained healthy at the ocular site (no effect size could be calculated). An effect size (or a Hedge's *g*) and variance were calculated to estimate the effect of MOMP vaccination on chlamydial load (at the ocular and urogenital sites) and disease (at the urogenital site) for both field trials (a total of 6 effect sizes; see Borenstein et al. 2009). A meta-regression was performed on the effect sizes using the MAd package (Viechtbauer 2010) in the statistical program R (v3.5.3; Team 2017) and an $I^2$ statistic was calculated to determine heterogeneity between studies for each measurement (as omnibus models; Borenstein et al. 2009). As control koalas in the field trial by Desclozeaux et al. (2017a) did not have measurements of IFNγ or IL17 expression, a comparison was made for these immune measurements using univariate methods (Figure 3.1b). As no evidence indicated differences between the two trials, the data collected from Waugh et al. (2016) and Desclozeaux et al. (2017a) were pooled to test hypothesized models with chlamydial load, disease, and immune response measurements taken at six months after the first chlamydial vaccination.

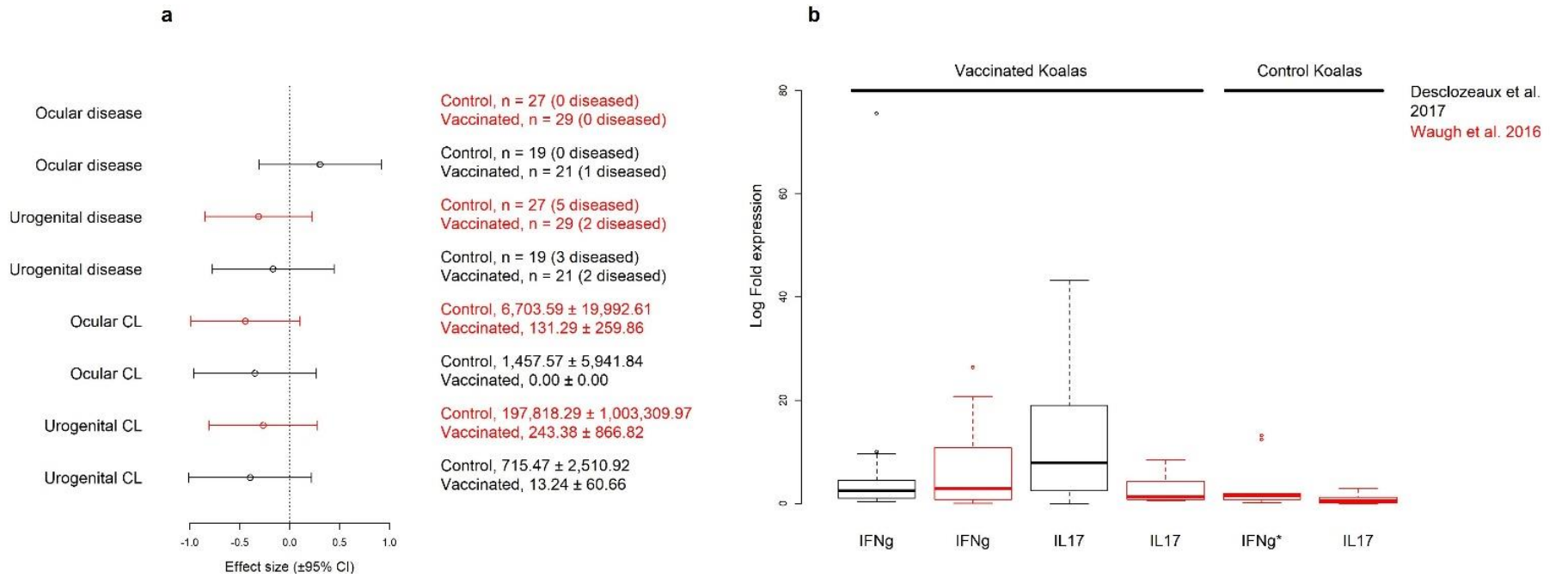
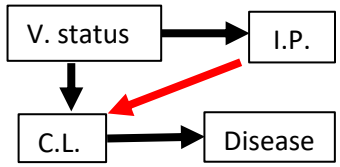
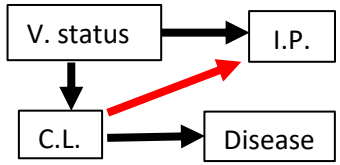
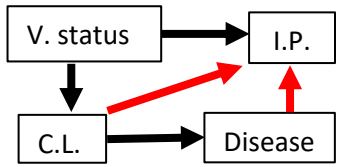
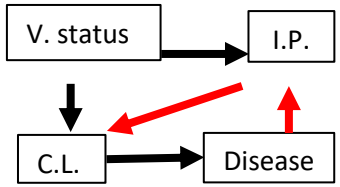
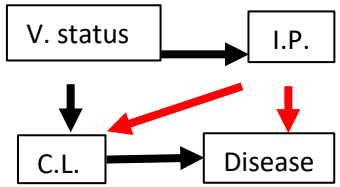
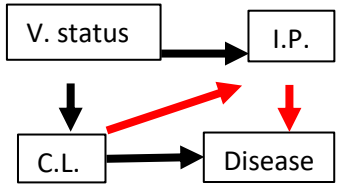



**Figure 3.1.** Comparison of hedge's g (±95% CI) effect sizes for disease and chlamydial load (CL) data collected at the urogenital and ocular sites for vaccinated and control groups (**a**), and $\log_{10}$ transformed interferon γ (IFNg) and interleukin 17 (IL17) cytokine expression for vaccinated and control groups (**b**) of wild koalas in two published MOMP-vaccination trials (see Appendix 3.1 and 3.2): Desclozeaux et al. 2017 (black effect sizes and boxplots) and Waugh et al. 2016 (red effect sizes and boxplots). All measurements were taken six months post vaccination. Sample size (with n diseased) or mean (± S.D.) copies/µL are shown for each factor in Figure 3.1a. Despite differences between the two trials, we found no evidence against pooling data between the two trials after performing a meta-regression comparing the effect of MOMP vaccination on disease and chlamydial load comparing treated and control koalas (meta-regression of effect size, g by field trial, p = 0.892). Immune cytokine measurements (fold gene expression relative to the housekeeping gene GAPDH) from vaccinated koalas six months post-vaccination were comparable between the two studies after performing a non-parametric Wilcoxon rank sum test for IFNg or IL17 (p=0.936, and p=0.075, respectively). To better visualise the data, the IFNg expression from one control koala (IFNg*) is excluded from the figure (2592.26 log fold expression), but included in all analyses. Note: only Waugh et al. 2016 measured the expression of both IFNg and IL17 from non-vaccinated control koalas. Additionally, no effect size could be estimated for ocular disease in the trial by Waugh et al. 2016 as all animals were clinically healthy at the ocular site.

### *3.3.3 Creation and testing of hypothesized models*

Six *a priori* structural equation models were created based on biological hypotheses proposed in the literature (Table 3.1; see Appendix 3.1 for additional background information and Appendix 3.2 for measurement sample sizes). We created our hypothesized models with the assumptions that vaccination affected chlamydial load and host immune parameters (black arrows between V. Status and either C.L. or I.P., see Table 3.1) established in the original publications by Waugh et al. (2016) and Desclozeaux et al. (2017a). We also made our hypothesized models with the assumption that MOMP vaccination was not directly associated with disease based on previous studies that evaluated the safety of vaccinating healthy koalas against *Chlamydia* (Hernández-Sánchez et al. 2015; Kollipara et al. 2012). Lastly, we assumed that there was a link between chlamydial load and chlamydial disease (black arrow between C.L. and Disease, see Table 3.1) based on structural equation models of non-vaccinated free-ranging koalas (Quigley et al. 2018).

**Table 3.1.** Six hypotheses of MOMP (major outer membrane protein) vaccination status (V. status), immune parameter (I.P.) mRNA expression, chlamydial load (C.L.), and disease with explanations for each hypothesized model. Different models contain causal relationships assessed in different directions as indicated by the red arrows. The expression of one immune parameter was tested: interferon gamma (IFNg), or interleukin 17 (IL17). All six hypotheses were tested using chlamydial load and disease data collected from either the ocular or urogenital sites (i.e. 24 different models were tested in total). Arrows indicate the directionality of the relationship. Black arrows are relationships tested in only one direction, and red arrows indicate relationships tested in both directions.

| Hypothesis | Model diagram | Biological hypothesis |
|---|---|---|
| 1 |  | Immune parameter expression affects chlamydial load.<br><br>Immune parameter expression and disease are unrelated. |
| 2 |  | Chlamydial load affects immune parameter expression.<br><br>Immune parameter expression and disease are unrelated. |
| 3 |  | Chlamydial load affects immune parameter expression.<br><br>Immune parameter expression up-regulated with disease. |
| 4 |  | Immune parameter expression affects chlamydial load.<br><br>Immune parameter expression up-regulated with disease. |
| 5 |  | Immune parameter expression affects chlamydial load.<br><br>Immune parameter expression leads to scarring and disease. |
| 6 |  | Chlamydial load affects immune parameter expression.<br><br>Immune parameter expression leads to scarring and disease. |

To begin, IFNγ or IL17 expression was evaluated as the immune parameter. Two models were made to determine if, 1) a causal relationship exists between these immune parameters and disease, and 2) the directionality between these immune parameters and chlamydial load (hypotheses 1 and 2). Four additional models were made to assess the directionality between 1) chlamydial load and these immune parameters, and 2) these immune parameters and disease (hypotheses 3, 4, 5, and 6). Only one immune parameter was included at a time in each of the six hypothesized models so as to maintain appropriate 10:1 sample size to variable ratios (MacCallum et al. 1999), resulting in 30 different models. The data were separated by either the urogenital or ocular site and tested in each hypothesized model, which resulted in a total of 60 models tested. The ocular models include ocular chlamydial load and disease status (e.g. ocular conjunctivitis), and the urogenital models include urogenital chlamydial load and disease status (e.g. cystitis or dilation of the ovarian bursae).

Prior to model testing, we chose to standardize data from all variables into a 0 to 1 scale to make all model coefficients between all variables comparable within each model. Binomial values were assigned to vaccination status (0 for non-vaccinated and 1 for vaccinated), and ocular and urogenital disease status (0 for clinical disease absent or subclinical and 1 for clinical disease present). Ocular and urogenital chlamydial load measurements were placed in one of three ordinal categories from 0 to 1 depending on an untransformed qPCR result similar to those used by Quigley et al. (2018): 1) samples with no detectable qPCR result were given a value of 0, 2) samples with ≤100 copies·(µL of swab diluent tested)$^{-1}$ were detectable but not quantifiable and given a value of 0.5, and 3) samples with >100 copies·(µL of swab diluent tested)$^{-1}$ were detectable and quantifiable and given a value of 1.

Finally, values for both IFNγ and IL17 expression were transformed to a 0 to 1 scale by dividing each IFNγ or IL17 log fold expression measurement by the maximum IFNγ or IL17 log fold expression measurement, respectively, for all koalas in both studies.

Structural equation modelling (SEM) requires an individual to have all measurements for each variable tested in the model. We first tested each of the 60 models for model fit using the fit indices Root Mean Square Error of Approximation (RMSEA, absolute fit index) and Confirmatory Fit Index (CFI, relative fit index) described by Kline (Kline 2015), and a Bollen-Stine Bootstrap for non-parametric model fit (100 bootstrap draws; Bollen and Stine 1992; Kim and Millsap 2014). No further analysis was performed for models that failed to fit the data using these indices (CFI > 0.9; RMSEA < 0.05; Bollen-Stine p-value > 0.05; Bollen and Stine 1992; Kline 2015). Models that fit the data, however, were then compared using Akaike's information criterion corrected for small sample sizes (AICc) and model weights ($w_i$) were calculated (Burnham and Anderson 2004; Burnham et al. 2011). All models were tested using R statistical software (v3.5.3; Team 2017) using the lavaan package (Rosseel 2012).

*3.3.4 Model interpretation*

A coefficient of determination ($R^2$) was obtained for disease, chlamydial load, and immune parameter expression for each of the fitting models to estimate how well the model predicts each of these variables within the model. All standardized relationship coefficients (± 95% CI) were obtained for each path tested in each of the fitting models. Standardized relationship coefficients indicate the strength of the relationship (the magnitude of the coefficient), direction of the relationship (e.g. positive relationships indicate positive effects of one variable on another), and how well each relationship is predicted (by the 95% CI) by

the model. We used standardized relationships in our model interpretation so we could compare the magnitude of all relationships within each model. As some models contained different sample sizes, care was taken not to compare relationship coefficients (or $R^2$) across unrelatable models (i.e. models that do not contain the same variables). We defined strong model relationships based on 95% CI and whether it crossed 0, the threshold of an effect of an exogenous (predictor) variable on an endogenous variable (response). As our models were similar in complexity and limited by sample size, it was not uncommon that more than one model fit the data. For multiple model interpretation, we used model averaging to calculate model averaged parameters (and variance, 95% CI) using the previously described model weights (see Burnham et al. 2011 for equation).

**3.4 Results**

We first investigated the effects of MOMP vaccination six months after the initial vaccination on chlamydial abundance and disease at two important mucosal sites (ocular and urogenital) between two *Chlamydia* vaccination trials using free-ranging koalas (Desclozeaux et al. 2017a; Waugh et al. 2016). We estimated effect sizes (as Hedge's g) for these responses (see Methods; Lizárraga et al. 2019) and found no evidence for a difference in MOMP vaccination on chlamydial load (ocular and urogenital) and disease (urogenital) between field trials (meta-regression, p=0.880; Figure 3.1a). A similar result was obtained when we analysed the effect of MOMP vaccination on disease (urogenital) and transformed chlamydial load data (urogenital and ocular being placed into one of three ordinal categories, see Methods; meta-regression, p=0.247). Estimations of heterogeneity were low for pairwise measurements of urogenital chlamydial load ($I^2$= 0.000), ocular chlamydial load ($I^2$= 0.000), and urogenital disease ($I^2$= 0.000). Additionally, we found measurements of IFNγ

or IL17 expression in vaccinated animals to be comparable between the two experiments after performing a non-parametric Wilcoxon rank sum test (IFNγ, p=0.936; IL17, p=0.075; Figure 3.1b). As we found no evidence supporting a difference between the two trials, we pooled the data to obtain sample sizes sufficient to use in structural equation models.

The pooled data from the two trials resulted in a large collection of data for MOMP vaccinated (n=50) or control (n=46) wild koalas with recorded clinical disease status (control or vaccinated with ocular disease n = 1, control or vaccinated with urogenital disease n = 7; see Appendix 3.2) and measured chlamydial load out to six months post-vaccination. Of these 96 koalas, a subset of animals had measurements of either IFNγ (n=40, n=25 vaccinated koalas, n=15 control koalas) or IL17 (n=36, n=25 vaccinated koalas, n=11 control koalas) expression (see Appendix 3.2), and those that were missing these measurements were removed from the analysis (n=51 and n=55 for IFNγ and IL17, respectively). Of the 40 animals in the IFNγ expression subset (inclusive of all animals in the subset with IL17 expression), 13 animals had an ocular chlamydial load of $\geq$100 copies·$\mu L^{-1}$ (n = 7 vaccinated, n = 6 control) at baseline, while 16 animals had an urogenital chlamydial load of $\geq$100 copies·$\mu L^{-1}$ (n = 7 vaccinated, n = 9 control) at baseline. As sample size was limited for this analysis, we could not exclude animals with an infection at baseline nor could we introduce another variable into our models. We focussed on models with both immune parameters analysed separately as structural equation modelling requires robust sample sizes (i.e. ratio of samples to variables analysed = 10:1, (MacCallum et al. 1999); Appendix 3.3). When we tested the 6 hypotheses for both the ocular and urogenital sites with either IFNγ or IL17 expression (Table 3.1), we obtained multiple models that fit the data (Appendix 3.4). For multiple hypothesis interpretation, we focussed on coefficients where the 95% confidence

interval did not overlap 0 (a strategy used in other structural equation modelling studies (such as Dorresteijn et al. 2015). Additionally, we used a model averaging approach to determine if the direction and magnitude of these relationships were consistent across multiple models.

*3.4.1 Models with ocular chlamydial load and disease: MOMP vaccination reduced ocular chlamydial load*

All six models with ocular chlamydial load, disease, and IFNγ expression fit the data (Appendix 3.4). We found that MOMP vaccination had a negative effect on chlamydial load in koalas (hypothesis 1, -0.286 ± 0.276; hyp. 2, -0.320 ± 0.300; hyp. 3, -0.320 ± 0.288; hyp. 6, -0.320 ±0.259; Table 3.2). We obtained the same result when all models were averaged together (model average, -0.303 ± 0.300; Table 3.2 and Figure 3.2a).

Four models containing ocular chlamydial load, disease, and IL17 expression fit the data (hypotheses 3, 4, 5, and 6; Appendices 3.4, 3.5, and 3.6). Two of these hypotheses showed that koalas receiving a MOMP vaccination tended to have a lower chlamydial load (hypothesis 3, -0.320 ± 0.304; hyp. 6, -0.320 ± 0.314; Table 3.2 and Figure 3.2c), agreeing with models containing IFNγ expression. Three hypotheses indicate that MOMP vaccinated koalas produced more IL17 compared to control koalas (hypothesis 3, 0.106 ± 0.065; hyp. 5, 0.166 ± 0.104; hyp. 6, 0.130 + 0.100), and ocular chlamydial load negatively predicted IL17 expression (-0.115 ± 0.102; hypothesis 6). Lastly, one hypothesis supported a relationship between ocular disease and an increase in IL17 expression (0.832 ± 0.080, hypothesis 3; see Appendix 3.5 for ocular disease raw data); this however, is complicated as only one koala developed ocular disease after six months (IL17 fold expression compared to glyceraldehyde 3-phosphate dehydrogenase, GAPDH = 43.21, n=1; see Appendix 3.5 for a comparison with

other IL17 expression measurements). When all models were averaged, only one relationship had a marginal effect (ocular chlamydial load has an inverse relationship with IL17 expression; -0.052 ± 0.052; Table 3.2 and Figure 3.2c).

**Table 3.2.** Standardized estimates (± 95% CI) for each relationship within all best fitting models identified (see Appendix 3.4 for model fit and weights). MOMP vaccination status (MVS), immune parameter (IP; either IL17 or IFNγ expression), disease (either urogenital or ocular), and chlamydial load (CL; either urogenital or ocular) were modelled to test the six hypotheses described in Table 3.1. Dashed lines represent untested relationships. Bold values represent strong relationships where a coefficient ± 95% confidence interval does not cross zero.

| Site | Immune parameter | Hypothesis /Model Average | MVS→CL | MVS→IP | CL→Disease | IP→CL | CL→IP | Disease→IP | IP→Disease |
|---|---|---|---|---|---|---|---|---|---|
| Ocular | IFNγ | 1 | **-0.286 ± 0.276** | -0.065 ± 0.137 | -0.043 ± 0.078 | 0.531 ± 30.064 | -- | -- | -- |
| | | 2 | **-0.320 ± 0.300** | -0.038 ± 0.084 | -0.043 ± 0.080 | -- | 0.083 ± 0.165 | -- | -- |
| | | 3 | **-0.320 ± 0.288** | -0.039 ± 0.071 | -0.043 ± 0.076 | -- | 0.084 ± 0.135 | 0.016 ± 0.024 | -- |
| | | 4 | -0.285 ± 0.547 | -0.066 ± 11.25 | -0.045 ± 0.304 | 0.538 ± 167.043 | -- | 0.024 ± 574.97 | -- |
| | | 5 | -0.286 ± 0.296 | -0.065 ± 0.114 | -0.044 ± 0.088 | 0.531 ± 41.195 | -- | -- | 0.007 ± 10.653 |
| | | 6 | **-0.320 ± 0.259** | -0.038 ± 0.080 | -0.044 ± 0.084 | -- | 0.083 ± 0.155 | -- | 0.007 ± 14.876 |
| | | Average | **-0.303 ± 0.300** | -0.052 ± 0.659 | -0.043 ± 0.091 | 0.266 ± 22.395 | 0.042 ± 0.081 | 0.002 ± 28.456 | 0.001 ± 1.250 |
| | IL17 | 3 | **-0.320 ± 0.304** | **0.106 ± 0.065** | -0.049 ± 0.080 | -- | -0.085 ± 0.089 | **0.832 ± 0.080** | -- |
| | | 4 | -0.253 ± 2.517 | 0.132 ± 8.159 | 0.010 ± 0.321 | -0.402 ± 20.800 | -- | 0.851 ± 296.338 | -- |
| | | 5 | -0.257 ± 0.343 | **0.166 ± 0.104** | 0.041 ± 0.057 | -0.379 ± 0.388 | -- | -- | 0.526 ± 0.604 |
| | | 6 | **-0.320 ± 0.314** | **0.130 ± 0.100** | 0.041 ± 0.057 | -- | **-0.115 ± 0.102** | -- | 0.526 ± 0.570 |
| | | Average | -0.290 ± 0.846 | 0.131 ± 2.021 | 0.006 ± 0.129 | -0.183 ± 5.092 | **-0.052 ± 0.052** | 0.457 ± 71.095 | 0.240 ± 0.305 |
| Urogenital | IFNγ | 3 | -0.253 ± 0.296 | -0.043 ± 0.080 | 0.347 ± 0.355 | -- | 0.066 ± 0.108 | 0.116 ± 0.274 | -- |
| | | 4 | -0.226 ± 0.251 | -0.060 ± 0.106 | 0.303 ± 0.416 | 0.429 ± 38.306 | -- | 0.120 ± 0.208 | -- |
| | | 5 | -0.213 ± 0.251 | -0.065 ± 0.135 | 0.267 ± 0.357 | 0.629 ± 26.074 | -- | -- | 0.645 ± 23.189 |
| | | 6 | -0.253 ± 0.261 | -0.037 ± 0.096 | 0.267 ± 0.412 | -- | 0.109 ± 0.216 | -- | 0.645 ± 24.712 |
| | | Average | -0.236 ± 0.265 | -0.051 ± 0.104 | 0.298 ± 0.386 | 0.263 ± 16.549 | 0.043 ± 0.080 | 0.063 ± 0.130 | 0.299 ± 11.176 |
| | IL17 | 3 | **-0.320 ± 0.261** | **0.112 ± 0.084** | **0.394 ± 0.361** | -- | **-0.244 ± 0.178** | 0.208 ± 0.257 | -- |
| | | 4 | -0.183 ± 0.300 | **0.193 ± 0.149** | **0.563 ± 0.537** | -0.821 ± 0.841 | -- | 0.241 ± 0.357 | -- |
| | | 5 | -0.241 ± 0.343 | **0.166 ± 0.110** | **0.541 ± 0.386** | -0.474 ± 0.541 | -- | -- | 0.688 ± 0.737 |
| | | 6 | -0.320 ± 0.321 | **0.115 ± 0.086** | **0.541 ± 0.335** | -- | **-0.161 ± 0.122** | -- | 0.688 ± 0.792 |
| | | Average | -0.269 ± 0.308 | **0.145 ± 0.107** | **0.506 ± 0.407** | -0.308 ± 0.398 | **-0.107 ± 0.084** | 0.114 ± 0.161 | 0.338 ± 0.435 |

Note: Model averages are determined using the model weights in Appendix 3.4 (see Methods for model weight equation).

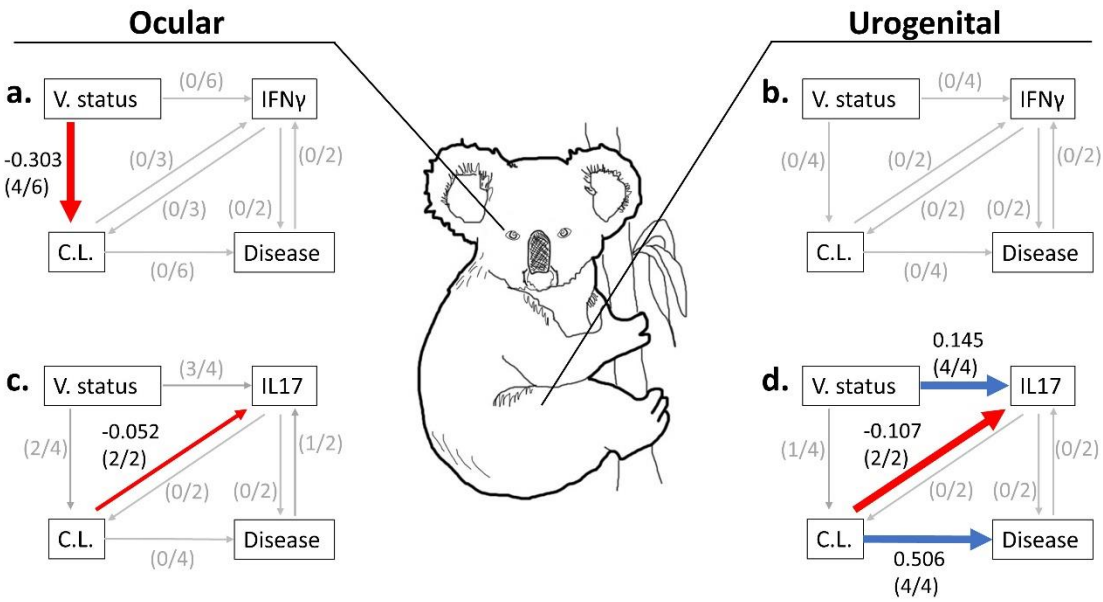



**Figure 3.2.** Summary figure of averaged models in Table 3.4 for ocular (**a** and **c**) and urogenital (**b** and **d**) data tested with either the expression of IFNγ (**a** and **b**) or IL17 (**c** and **d**) six months post-vaccination. Red and blue lines represent relationships with a negative or positive coefficient, respectively, where the coefficient and its variance (95% CI) did not cross 0 (i.e. the threshold of effect). Very small gray lines represent tested relationships in the averaged model where coefficients and its variance (95% CI) crossed 0. The number of hypotheses with a clear relationship (where the coefficient and its 95% CI did not cross 0) between two variables are listed in parentheses. Note: Care should be made in the interpretation of this summary figure as it does not represent the results from any single model, but across all best fitting models using a model averaging approach. The directionality and magnitude of each coefficient shown in this figure are supported by significant coefficients (i.e. coefficients with 95% CIs that do not cross 0) obtained from the averaged models in Table 3.2. V. status = MOMP vaccination status, C.L. = chlamydial load, Disease = chlamydial disease

*3.4.2 Models with urogenital chlamydial load and disease: MOMP vaccinated koalas reduced chlamydial load and had more IL17 gene expression compared to control animals*

Four models with urogenital chlamydial load, disease, and IFNγ expression that fit the data (hypotheses 3, 4, 5, and 6; Appendices 3.4, 3.6, and 3.7). When we investigated the coefficients for each relationship across all urogenital models with IFNγ expression, we found no clear relationships due to the large variance for each coefficient. As a result, we found no clear support for directionality or magnitude between IFNγ expression and urogenital chlamydial load or IFNγ expression and urogenital disease, though some relationships within these models were trending. We found a trending relationship between MOMP vaccinated koalas and a lower urogenital chlamydial load compared to control koalas (-0.236 ± 0.265, Table 3.2 and Figure 3.2b).

There were four hypotheses with urogenital chlamydial load and disease and IL17 expression that fit the data (hypotheses 3, 4, 5, and 6; Appendix 3.4). There was clear evidence that MOMP vaccinated koalas had a lower urogenital chlamydial load compared to control koalas (hypothesis 3, -0.320 ± 0.261). MOMP vaccinated koalas produced more IL17 compared to control animals that was supported by all four hypotheses (hypothesis 3, 0.112 ± 0.084; hyp. 4, 0.193 ± 0.149; hyp. 5, 0.166 ± 0.110; hyp. 6, 0.115 ± 0.086; Table 3.2). These four hypotheses also indicate that diseased koalas typically had a high chlamydial load (hypothesis 3, 0.394 ± 0.361; hyp. 4, 0.563 ± 0.537; hyp. 5, 0.541 ± 0.386; hyp. 6, 0.541 ± 0.335; Table 3.2). Two hypotheses indicate that when all koalas are considered (all 36 koalas from both the control or vaccinated groups) with a high chlamydial load tended to produce less IL17 compared to koalas with a low chlamydial load (hypothesis 3, -0.244 ± 0.178; hyp. 6, -0.161 ± 0.122; Table 3.2). Lastly, across all hypotheses, we found no relationship

between IL17 expression and urogenital disease. When all urogenital models with IL17 expression were averaged we found: 1) that MOMP vaccinated koalas produced more IL17 compared to control koalas (0.145 ± 0.107), 2) koalas with a high chlamydial load were more likely to develop urogenital disease six months post-vaccination (0.506 ± 0.407), and 3) koalas (both vaccinated and control) with a high urogenital chlamydial load tended to express less IL17 mRNA compared to koalas with a lower urogenital chlamydial load (-0.107 ± 0.084; Table 3.2 and Figure 3.2d). These structural equation models therefore connect relationships among MOMP vaccination, IL17 expression, urogenital chlamydial load, and disease (Figure 3.2d).

**3.5 Discussion**

There exist many unknown aspects surrounding chlamydial vaccination in koalas primarily due to the difficulty in obtaining samples and the cost for vaccine experiments. Much of the underlying chlamydial biology, particularly complex immunological responses to infections, is understood from experiments using the mouse model or from *in vitro* cell cultures. In the current study, data from two free-ranging koala vaccination trials (both using the major outer membrane protein, MOMP) were used to investigate the effects of MOMP vaccination both directly and indirectly on the expression of koala immune parameters, chlamydial infection, and disease. Koalas were compared from either vaccinated or control groups to evaluate vaccine efficacy given variability associated with free-ranging outbred animals (see Methods). Despite sources of variation in our data (numbers of koalas, use of outbred animals, unknown number of sexual encounters, unknown infection dates) there were clear relationships identified in our analyses that provide unique insights into the effects of vaccinating free-ranging koalas against *Chlamydia*, relative to unvaccinated

individuals in the population. In particular, this study has helped establish one immune cytokine, IL17, may be an important factor in MOMP vaccinated koalas, however, animals with high chlamydial loads are expressing low amounts of IL17 and are more likely to develop urogenital disease.

Two measurements of the koala immune response were incorporated into the models created in this study, IFNγ and IL17 expression. IFNγ, a T-cell secreted cytokine, is currently regarded as one of most important cytokines in chlamydial biology (Brunham and Rey-Ladino 2005). Across all pre-clinical studies investigating a chlamydial vaccine using various animal models (mice, non-human primates, cats, pigs, guinea pigs etc.), there exists a common aim of eliciting strong mucosal T-cell responses, particularly increases to IFNγ concentration (Lizárraga et al. 2019; Phillips et al. 2019). Though IFNγ is involved in multiple immune pathways, IFNγ production leads to the enzymatic degradation of tryptophan, effectively starving *Chlamydia* of this essential amino acid (Vasilevsky et al. 2014). Some studies suggest that tryptophan starvation may even cause *C. trachomatis* to enter a persistence form (Beatty et al. 1994; Beatty et al. 1993). Experiments conducted *in vitro* show that elevated IFNγ concentrations prevent chlamydial growth in *C. trachomatis*, but not *C. pecorum* (Islam et al. 2018). To our knowledge only one study, beside the two reported in the current study, has reported measurements of IFNγ expression and chlamydial load after MOMP vaccination in diseased koalas *ex vivo*. Nyari et al. (2019) found that vaccination of captive koalas with MOMP (delivered along with Tri-adjuvant) did not have a clear effect on systemic IFNγ expression *ex vivo* despite a reduction in ocular disease among seven koalas (pre-vaccination compared to 6 weeks post vaccination, n=7). It is certainly possible that *C. trachomatis* and *C. pecorum* have differing sensitivities to IFNγ

which may explain why measurements of koala IFNγ expression had no relationship with chlamydial load in any of our models (either ocular or urogenital; Table 3.2 and Figure 3.2a and 3.2b). These measurements of IFNγ expression contain large amounts of variance that can be seen in the raw data (Appendix 6). The average log transformed IFNγ measurement between the non-vaccinated and vaccinated groups were similar, 1.378 ± 0.918 (95% CI, n=16) and 1.445 ± 0.430 (95% CI, n=25), respectively. Additionally, IFNγ measurements have been shown to be dependent on the stage of chlamydial infection in mice (Cheng et al. 2014) and non-human primates (Cheng et al. 2011), something that is currently uncontrollable in trials using free-ranging koalas. More longitudinal studies are needed to determine if IFNγ expression is a key factor in protection against *C. pecorum* infection in free-ranging koalas.

In contrast, models with IL17 expression indicated that koalas that typically had higher ocular and urogenital chlamydial load tended to have low levels of IL17 expression, six months post-vaccination (Table 3.2 and red arrows in Figure 3.2c and 3.2d). Experiments using IL17 knockout mice have shown that IL17 is a factor in both protection (Scurlock et al. 2011) as well as pathogenesis (Andrew et al. 2013). IL17 is a pro-inflammatory cytokine secreted mainly from T cells and has many functions in response to chlamydial infection. In the wild type mouse model and *in vitro* experiments, IL17 has been shown to work synergistically with IFNγ to inhibit *C. muridarum* growth by increasing the expression of inducible nitric oxide synthase (iNOS) and indirectly increasing antimicrobial nitric oxide (promoted by iNOS; Zhang et al. 2012). Indeed, iNOS has been shown to be up-regulated in human patients suffering from ectopic pregnancies associated with *C. trachomatis* infection (Refaat et al. 2009). In koalas, one study reported the IL17a response from koala PBMCs to

be higher in clinically diseased animals (n=12) compared to subclinical animals (n=29; Mathew et al. 2013). Three studies since have measured free-ranging koala IL17 response to chlamydial vaccination. In the first, Khan et al. (2016) focussed on non-diseased koalas that tested negative for *Chlamydia*, finding that IL17 expression increased with MOMP vaccination when compared to pre-vaccination levels. The second and third, Waugh et al. (2016) and Desclozeaux et al. (2017a), were included in our current analysis. One additional study using captive koalas with ocular disease reported comparable measurements of IL17a expression between koalas at pre-vaccination and six weeks post vaccination (n=7), even though there was improvement of ocular disease scores among the cohort at six weeks post vaccination (Nyari et al. 2019). In our current analysis, we found that koalas vaccinated with MOMP tended to have higher IL17 expression six months post-vaccination compared to non-vaccinated koalas in models based on urogenital data (Figure 2d). This was supported by three hypotheses (out of four) based on ocular data (Table 3.2). This increase can be seen by inspecting the raw data as the average log transformed expression of IL17 was higher in vaccinated compared to non-vaccinated groups, 1.616 ± 0.430 (95% CI, n=25) and 0.462 ± 0.279 (95% CI, n=12), respectively (Appendix 3.6). Additionally, we found no clear evidence for a direct relationship between IL17 expression and either ocular or urogenital disease that developed six months post-vaccination. Interestingly, the directionality of the relationship between chlamydial load and IL17, suggests that IL17 expression is dependent on chlamydial load. Perhaps animals with a high chlamydial load are unable to express IL17 or, more likely, are producing an immune response without IL17 when a high abundance of *Chlamydia* is present. Indeed, IL17 is one immune variable among many others that are part of a complex immune response to *Chlamydia* and its expression may or may not be a major factor in reducing chlamydial growth and preventing pathogenesis. More work needs to be

done to determine if IL17 expression is linked to the reduction of chlamydial load and protection against disease in koalas.

Both Waugh et al. (2016) and Desclozeaux et al. (2017a) evaluated the effect of MOMP vaccination on subsequent chlamydial disease, using univariate statistical methods. Waugh et al. (2016) found that fewer koalas developed disease after 12 months in the MOMP vaccinated group (1 of 23 koalas) compared to control animals (4 of 27 koalas). This result, however, was not statistically significant ($X^2$ = 1.512, p=0.363) likely owing to the fact that many chlamydial infections remain subclinical and that the total sample size (n=50) may have been too small to determine a statistically significant effect of vaccination on disease. Desclozeaux et al. (2017a) found that fewer koalas in the MOMP vaccinated group developed chlamydiosis (either ocular or urogenital chlamydial disease) compared to control koalas (0 of 21 and 3 of 21 for MOMP and control koalas, respectively); this comparison showed a trending (p<0.1) effect between groups ($X^2$ = 2.813, p=0.093). Bommana et al. (2017) also found that six MOMP vaccinated koalas, with a detectable chlamydial infection recorded at pre-vaccination, reduced their chlamydial load (in either the ocular, urogenital or both sites) 6-months post-vaccination (6 of 21 koalas), and this effect was statistically significant compared to control koalas (0 of 21 koalas; p=0.048). Lastly, our averaged models did not support a strong therapeutic effect (see Figure 2). This suggests that conservation efforts should be aimed at protecting uninfected individuals against future infection via vaccination, rather than vaccinating individuals who are already infected.

By combining data from both trials, we were able to expand upon these analyses by introducing additional realistic complexity using structural equation models. Preliminary

structural equation models indicated that a direct relationship between MOMP vaccination status and subsequent disease development failed our fitting criterion. Given this result, we created models where MOMP vaccination status indirectly affected disease status six months post-vaccination through direct relationships with either chlamydial load or an immune parameter. Ideally, we would have included both IFNγ and IL17 expression in the same models, but sample size limitations (having individual koalas in each group measured for all variables: IFNγ, IL17, infection load, disease status) prevented models with this level of complexity and, hence, we adopted a IFNγ or IL17 expression SEM approach. An additional ramification of our sample size limitation was that we were unable to exclude animals with baseline infections (animals with an ocular chlamydial load of >100 copies·$\mu L^{-1}$, n = 7 vaccinated and n = 6 control; or urogenital chlamydial load of >100 copies·$\mu L^{-1}$ n = 7 vaccinated and n = 9 control) nor were we able to include this as a variable within our models. Multiple models using ocular data and one model using urogenital data (Table 3.2) indicated that MOMP vaccination status had a direct negative effect on chlamydial load. These effects, however, were lost when we averaged all the urogenital models together. It is possible that given a larger sample size, we could have detected an effect. We performed a power analysis to determine the necessary sample size to detect a univariate effect of MOMP vaccination on urogenital chlamydial load. After estimating an effect size (Cohen's d) to estimate the effect of MOMP vaccination on urogenital chlamydial load (Borenstein et al. 2009), we found that a sample size of 117 koalas would be necessary to have an 80% chance of detecting an effect at a significance level of 0.05. This finding suggests that an effect might be obtained if a large-scale study included nearly three times the number of koalas that were used in our analysis. Additionally, the models created in this chapter contained two immune variables (one in each model) across two sites. While there was not a direct

association between chlamydial load and vaccination in this study, one might expect a different result if a different immune parameter were included in the model. Indeed, there exist a number of immune parameters whose expression could indirectly lead to the reduction of chlamydial load. Lastly, many publications have reported MOMP vaccinations have elicited protective effect (reviewed by Phillips et al. 2019), thus making this a viable conservation strategy in protecting naïve individuals from future infection.

In this study, we adopted a cross-sectional experimental design that focussed on control versus vaccinated koalas at six months post vaccination from which to implement our models. We chose this study design because research has shown koalas can become infected with *C. pecorum* and exhibit disease year-round. Therefore, background variation in individual parameters (immune factors, genotype, season sampled, etc.) appears insufficient to protect koalas from infection or disease, and the effect of a vaccine on immune parameters would most likely need to exceed normal background variation observed. We also acknowledge other valid study designs could be utilized. A common design for data similar to ours is to compare the change in variables over time (pre to post vaccination) between the control and vaccinated groups. Although this is a valid approach, in our study system we chose not to adopt the change in variable approach for the reason described above, and because a vaccination effect (relative to controls) could be observed at the cohort level and still be within background variation. While not the determining reason, we do also acknowledge that utilizing a change in chlamydial load or host immune expression from baseline would further reduce the sample size for analyses (n=3 for each model) in our study. Future research with a greater sample size of koala could consider a comparison of

these designs, or the extent to which individual responses to vaccination over time are repeatable.

Chlamydial load is among one of several factors that affect disease pathogenesis in free-ranging koalas (Quigley et al. 2018). Specifically, Quigley et al. (2018) found that measurements of chlamydial load (separated into 3 ordinal categories of severity) are strong predictors of urogenital disease, but not ocular disease. In our current study, we also found that koalas that developed urogenital chlamydial disease had a higher chlamydial load as compared to non-diseased animals when the data were modelled with IL17 expression. We did not detect this effect in models of ocular chlamydial load and disease, though this was likely due to a low prevalence of koalas with ocular disease (n=1). Previous studies have suggested that disease pathogenesis in koalas is complex (Madden et al. 2018; Wan et al. 2011). Three out of four of our averaged models (Figure 3.2b, 3.2c, and 3.2d) excluded Hypotheses 1 and 2, possibly suggesting that immune parameter and disease are related, however this remains unclear as only one relationship was significant (ocular chlamydial load and IL17; see Table 3.2). A large amount of variation in the prevalence of urogenital disease six months post-vaccination remains unexplained from even our best models ($R^2$=0.268). Including more factors that are currently associated with disease pathogenesis, including koala retrovirus (KoRV) status, may improve the predictability of these models.

**3.6 Conclusions**

In this study, we used structural equation models to identify direct and indirect relationships underpinning vaccination against chlamydial disease in free-ranging koalas. The use of structural equation modelling has previously been applied to identify complex factors influencing disease pathogenesis in koalas (Quigley et al. 2018). One factor, urogenital

chlamydial load, was positively associated with urogenital disease prevalence, agreeing with the findings of Quigley et al. (2018). We found that MOMP vaccination may indirectly affect the development of urogenital disease by increasing IL17 expression in koalas that inhibits the growth of *Chlamydia* (i.e. lower chlamydial load compared to control koalas). IL17 expression is one immune parameter that was increased in MOMP vaccinated koalas as compared to control koalas. Chlamydial load was a negative predictor of IL17 expression, suggesting that hosts express this cytokine less in response to chlamydial growth. Collectively, the structural equation models in this study highlight the importance of considering vaccine-immune-chlamydial load-disease relationships in a single analysis that can be performed in future studies of novel correlates of protection against *Chlamydia*. Lastly, more studies using free-ranging animals may be needed to determine the effect of multiple immune parameters on chlamydial load and disease and may make it possible to detect a direct effect of MOMP vaccination on chlamydial load.

**Chapter 3 Supplementary Material**

**Appendix 3.1.**

*Methods from Waugh et al. (2016) and Desclozeaux et al. (2017)*

Waugh et al. (2016) prepared a vaccine consisting of purified recombinant *Chlamydia pecorum* major outer membrane protein (MOMP) genotypes A, F, and G (50 µg of each antigen) combined with Immunostimulating Complex (ISC) adjuvant (50 µg). Sixty animals were randomly assigned to either a vaccinated (n=30) or control group (n=30) in the study in the Moreton Bay Region, Queensland, Australia from 2012 to 2015. Vaccinated koalas received three subcutaneous vaccinations over three months at one-month intervals. All

animals were assessed by a veterinarian before vaccination (i.e. day 0) and approximately six months post-vaccination for signs of chlamydial disease. During these assessments ocular and urogenital swabs were collected to detect *Chlamydia* by qPCR, and blood samples were collected to detect serum immunoglobulin G (IgG) or to measure the expression of interferon gamma (IFNγ) and interleukin 17 (IL17) using peripheral blood mononuclear cells (PBMCs) relative to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). A single housekeeping gene, GAPDH, was used as the reference for gene expression as previous studies have shown that the expression of this gene remains relatively stable after stimulation with either mitogens (Maher and Higgins 2016; Sarker et al. 2018) or chlamydial antigen (UV-inactivated *C. pecorum* G; Mathew et al. 2013; Mathew et al. 2014). The measurements of IFNγ and IL17 expression of mRNA were not published and the methods not described by Waugh et al. (2016). The methodology for measuring IFNγ and IL17 gene expression are, however, outlined in detail by Mathew et al. (2013) for IFNγ and by Mathew et al. (2014) for IL17. Both IFNγ and IL17 were normalised to GAPDH by the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001), #0}: $2^{-\Delta\Delta CT}$, where ΔΔCT is (threshold cycle (CT) for Cytokine expression – CT for GAPDH expression after 12 hours) – (CT for Cytokine expression – CT for GAPDH expression at time zero). Measurements of serum IgG were collected for 28 koalas (11 vaccinated and 17 control koalas), and an *in vitro* neutralisation assay was conducted for 9 control koalas. Due to the small number of koalas with measurements of IgG or *in vitro* neutralisation, we excluded these from further analysis in structural equation models (see Methods). Of the sixty koalas in this trial, 22 had both IFNγ and IL17 expression measurements. Additionally, 4 individuals had a measurement of IFNγ expression, but no measurement of IL17 expression (see Appendix 1a for the number of vaccinated and control groups). Of these subsets (26 and 22 koalas for IFNγ and IL17

expression, respectively), three of the control koalas and two of the vaccinated koalas had a urogenital disease diagnosis at six months post vaccination. There were no cases of ocular disease in either the vaccinated or control groups at six months post vaccination (see Appendix 3.1a).

The vaccine used by Desclozeaux et al. (2017a) consisted of purified recombinant major outer membrane protein (MOMP) genotypes A, F, and G (50 µg of each antigen) or peripheral membrane protein (PMP) genotype G, combined with tri-adjuvant consisting of PCEP (250 µg of poly[di(sodium carboxylatoethylphenoxy)]-phsophazene), IDR (500 µg), and polyI:C (250 µg). Sixty-three koalas from the Moreton Bay Region (different individuals from those vaccinated by Waugh et al. (2016) from 2013 to 2016 were randomly assigned to one of three groups: MOMP vaccinated (n=21), PMP vaccinated (n=21), or control (non-vaccinated; n=21). All animals were assessed by a veterinarian before vaccination (i.e. day 0) and approximately six months after the first vaccination for signs of chlamydial disease. Swabs were collected at the ocular and urogenital sites from all animals to determine chlamydial load by qPCR, but blood samples were only taken from vaccinated individuals for measurements of IFNγ and IL17 as previously described. Control animals from this trial did not have measurements of systemic cytokine expression. Of the 42 koalas in this trial that could be included in our analysis (i.e. PMP vaccinated individuals excluded), 14 vaccinated koalas had both IL17 and IFNγ expression measurements. There were no measurements of either IL17 or IFNγ expression for control koalas (see Appendix 3.1a). Of the 14 vaccinated koalas six months post vaccination, two koalas had a urogenital disease diagnosis and one koala had an ocular disease diagnosis.

Two individuals were included as controls in both the study by Waugh et al. (2016) and Desclozeaux et al. (koalas were named Nyx and Teena; Desclozeaux et al. 2017a). Both koalas were included only once in our study. We used the data reported by Waugh et al. (2016) for these animals in our models (excluding any data for these koalas by Desclozeaux et al. 2017a) as cytokine expression measurements were taken for control koalas.

*Biological Hypotheses*

In addition to the number of assumptions we made, we were interested in testing a number of hypothesized relationships between 1) host immune parameter and chlamydial load (hypotheses 1, 4, and 5), 2) chlamydial load and host immune parameter (hypotheses 2, 3, and 6), 3) immune parameter and disease (hypotheses 5 and 6), and 4) disease and host immune parameter (hypotheses 3 and 4). We chose hypotheses linking host immune parameter and chlamydial load (hypotheses 1, 4, and 5) as there is evidence that IFNγ is linked to chlamydial load reduction *in vitro* (Beatty et al. 1993) and we hypothesized the expression of a host immune parameter would suppress chlamydial load. We tested this hypothesis in the opposite direction (hypotheses 2, 3, and 6) to determine if animals having a small chlamydial abundance was a predictor of producing more of a given host immune parameter. We tested immune parameter and disease (hypotheses 5 and 6) as IL17 expression was previously hypothesized to be a cause of disease in koalas (Mathew et al. 2014). Lastly, we tested this relationship in the reverse direction (hypotheses 3 and 4) to determine if diseased koalas are more likely to upregulate host immune parameters.

**Appendix 3.2.** Study summaries of Waugh et al. 2016, Desclozeaux et al. 2017 and a total combining koalas or measurements from both studies. Pooled group sizes for structural equation modelling containing either IL17 or IFNγ mRNA expression include control and vaccinated koalas with complete data only (i.e. individuals with missing measurements were excluded). The bold numbers represent sample sizes for structural equation models in this study. MOMP = major outer membrane protein, PMP = peripheral membrane protein

| Study summary or group size | Waugh et al. 2016 | Desclozeaux et al. 2017 | Total |
|---|---|---|---|
| Years sampled (all seasons) | 2012 to 2015 | 2014 to 2016 | -- |
| Geographic location of trial | Moreton Bay, Queensland | Moreton Bay, Queensland | -- |
| Vaccine(s) tested | MOMP | MOMP, PMP | -- |
| Total number of koalas in trial | 60 | 63 | 123 |
| Number of unique MOMP vaccinated koalas | 30 | 21 | 51 |
| Number of unique control koalas | 30 | 19* | 51 |
| Cytokine measurements for vaccinated koalas? | Yes | Yes | -- |
| Number of koalas with an IL17 expression measurement (vaccinated) | 11 | 14 | 25 |
| Number of koalas with an IFNγ expression measurement (vaccinated) | 11 | 14 | 25 |
| Cytokine measurements for control koalas? | Yes | No | -- |
| Number of koalas with an IL17 expression measurement (control) | 11 | 0 | 11 |
| Number of koalas with an IFNγ expression measurement (control) | 15 | 0 | 15 |
| Pooled number of vaccinated and control koalas for a model with IL17 expression | 22 | 14 | **36** |
| Pooled number of vaccinated and control koalas for a model with IFNγ expression | 26 | 14 | **40** |
| Number of pooled vaccinated and control koalas with urogenital disease at 6 months post vaccination** | 5 | 2 | 7 |
| Number of pooled control and vaccinated koalas with ocular disease at 6 months post vaccination** | 0 | 1 | 1 |

*Two koalas (Nyx and Teena) were included as control koalas in both the trial by Waugh et al. 2016 and Desclozeaux et al. 2017. We included these individuals only once (included in the Waugh et al. 2016 column in the table), thus 19 individuals are reported in the column for Desclozeaux et al. 2017.
**Both pooled groups of vaccinated and control koalas that contain either IFNγ (n=40) or IL17 expression (n=36) have the same number of diseased individuals

**Appendix 3.3.** Sample size of MOMP-vaccinated koalas (data pooled for Waugh et al. 2016 and Desclozeaux et al. 2017) for each modelled variable and the number of koalas that have measurements for all variables (required for structural equation modelling) as models are incrementally increased in complexity. Two variable models can be analysed using single predictor generalized linear models, but would be less informative compared to structural equation models that require robust sample sizes. Bold terms are sample sizes used in models in this study. C.L. = chlamydial load; IFNγ = interferon gamma mRNA expression; IL17 = interleukin 17 mRNA expression

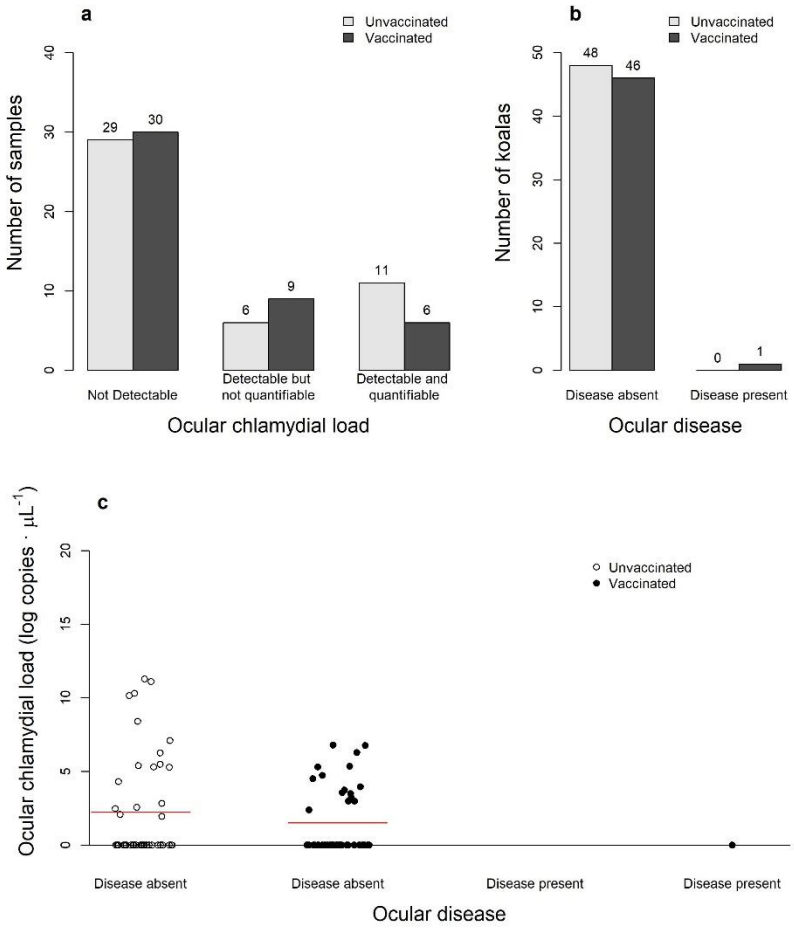
| Variables in model | Sample size for urogenital or ocular sites (n) | Suitable for SEM? |
|---|---|---|
| Vaccination status, Disease | 93 | No* |
| Vaccination status, C.L. | 89 | No* |
| Vaccination status, IFNγ | 41 | No* |
| Vaccination status, IL17 | 37 | No* |
| **Vaccination status, Disease, C.L., IFNγ** | **40** | **Yes** |
| **Vaccination status, Disease, C.L., IL17** | **36** | **Yes** |
| Vaccination status, Disease, C.L., IFNγ, IL17 | 36 | No** |

*Use of a generalized linear model (GLM) is more suitable.

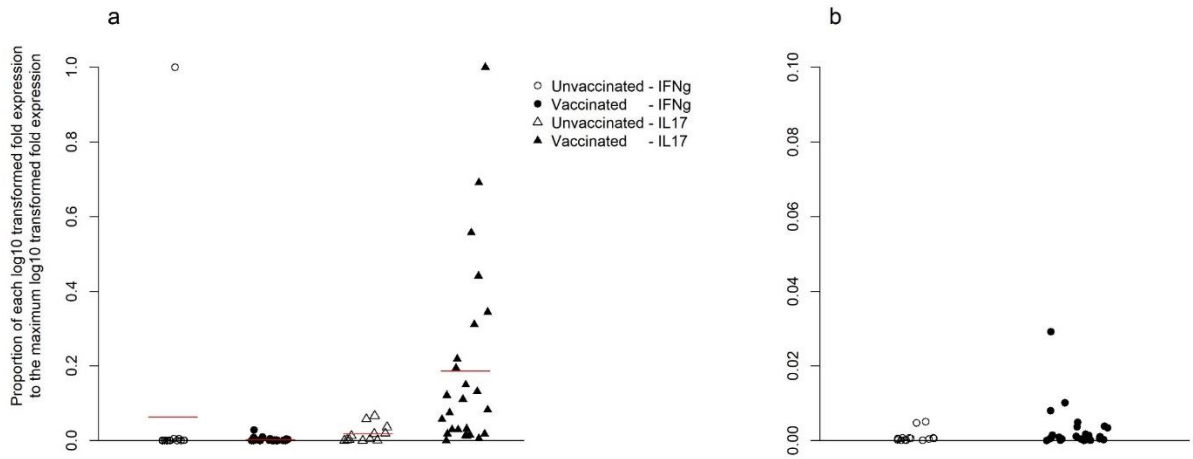
**Insufficient sample size.

**Appendix 3.4.** Results of model fitting testing the six hypotheses shown in Table 3.1. Improvement in the model fit is listed as the change in Akaike's information criterion with a correction for small sample sizes (ΔAICc) and model weight ($w_i$), along with a coefficient of determination ($R^2$) for disease, chlamydial load (C.L.), and expression of an immune parameter used in the model. Dashes lines indicate models that did not fit the data and were not compared using AICc.

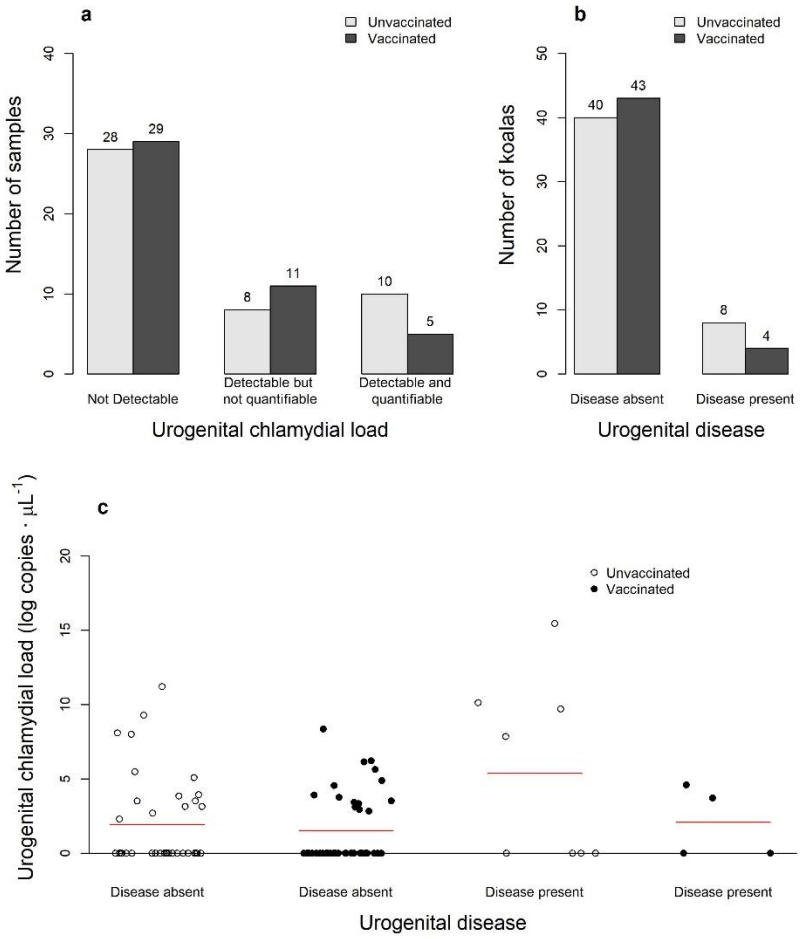
| Site | Immune parameter | Hypothesis | Did the model fit? | ΔAICc | $w_i$ | Disease $R^2$ | C.L. $R^2$ | Immune parameter $R^2$ |
|---|---|---|---|---|---|---|---|---|
| Ocular | IFNγ | 1 | Yes | 0.000 | 0.402 | 0.013 | 0.177 | 0.040 |
| | | 2 | Yes | 0.000 | 0.402 | 0.013 | 0.139 | 0.083 |
| | | 3 | Yes | 4.200 | 0.049 | 0.013 | 0.139 | 0.083 |
| | | 4 | Yes | 4.188 | 0.049 | 0.013 | 0.177 | 0.041 |
| | | 5 | Yes | 4.210 | 0.049 | 0.013 | 0.177 | 0.040 |
| | | 6 | Yes | 4.210 | 0.049 | 0.013 | 0.139 | 0.083 |
| | IL17 | 1 | No | -- | -- | -- | -- | -- |
| | | 2 | No | -- | -- | -- | -- | -- |
| | | 3 | Yes | 0.000 | 0.304 | 0.014 | 0.137 | 0.536 |
| | | 4 | Yes | 0.477 | 0.240 | <0.001 | 0.164 | 0.503 |
| | | 5 | Yes | 0.575 | 0.228 | 0.456 | 0.174 | 0.123 |
| | | 6 | Yes | 0.575 | 0.228 | 0.456 | 0.137 | 0.161 |
| Urogenital | IFNγ | 1 | No | -- | -- | -- | -- | -- |
| | | 2 | No | -- | -- | -- | -- | -- |
| | | 3 | Yes | 0.077 | 0.263 | 0.125 | 0.180 | 0.101 |
| | | 4 | Yes | 0.000 | 0.273 | 0.123 | 0.157 | 0.157 |
| | | 5 | Yes | 0.324 | 0.232 | 0.188 | 0.162 | 0.040 |
| | | 6 | Yes | 0.324 | 0.232 | 0.188 | 0.101 | 0.106 |
| | IL17 | 1 | No | -- | -- | -- | -- | -- |
| | | 2 | No | -- | -- | -- | -- | -- |
| | | 3 | Yes | 0.000 | 0.304 | 0.143 | 0.150 | 0.310 |
| | | 4 | Yes | 0.478 | 0.240 | 0.114 | 0.170 | 0.114 |
| | | 5 | Yes | 0.576 | 0.228 | 0.268 | 0.215 | 0.123 |
| | | 6 | Yes | 0.576 | 0.228 | 0.268 | 0.190 | 0.150 |




**Appendix 3.5.** Sample size for koala ocular chlamydial load placed in ordinal categories used in structural equation models (a), ocular disease status (b), and log transformed chlamydial load measurements were plotted against disease status (c). Light bars and unfilled symbols represent non-vaccinated koalas, and dark bars and filled symbols represent MOMP-vaccinated koalas. Chlamydial load qPCR values were grouped ordinally (based on untransformed measurements) such that samples with no detectable qPCR result were "not detectable", samples with ≤100 copies·µL$^{-1}$ were "detectable but not quantifiable", and samples with >100 copies·µL$^{-1}$ were "detectable and quantifiable". Red lines indicate the mean value for each group.




**Appendix 3.6.** Proportion of each log transformed fold gene expression value to the maximum log transformed fold gene expression value for both interferon γ (IFNg; circles) or interleukin 17 (IL17; triangles) in unvaccinated (unfilled symbols) or MOMP-vaccinated (filled symbols) free-ranging koalas (a) and IFNg expression (in both vaccinated and unvaccinated animals with a limited y-axis (one exceptional point is not visible) of individual IFNg expression values (b). Gene expression of IFNg or IL17 was measured from koala PBMCs collected six months post-vaccination. Cells were stimulated with *Chlamydia pecorum* elementary bodies and expression was compared to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Red lines indicate the mean value for each group.




**Appendix 3.7.** Sample size for koala urogenital chlamydial load placed in ordinal categories used in structural equation models (a), urogenital disease status (b), and log transformed chlamydial load measurements were plotted against disease status (c). Light bars and unfilled symbols represent non-vaccinated koalas, and dark bars and filled symbols represent MOMP-vaccinated koalas. Chlamydial load qPCR values were grouped ordinally (based on untransformed measurements in copies·µL$^{-1}$) such that samples with no detectable qPCR result were "not detectable", samples with ≤100 copies·µL$^{-1}$ were "detectable but not quantifiable", and samples with >100 copies·µL$^{-1}$ were "detectable and quantifiable". Red lines indicate the mean value for each group.

## Chapter 4: The variability of individual immune responses to vaccination: a koala-*Chlamydia* case study

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## 4.1 Abstract

For researchers and practitioners, an ideal property of vaccines is that they elicit predictable immune responses with minimal variation among individuals. Many studies examine the average effect of vaccines under development on a group (a cohort effect), but explicit analysis of individual responses is less common. In this study, the data from three *Chlamydia* vaccine studies of koalas (Waugh et al. 2016; Desclozeaux et al. 2017; and Nyari et al. 2019) which showed average increases of IgG (immunoglobulin G) were evaluated. The individual variability of IgG measurements was assessed using mixed-effects methods adapted from the behaviour literature. This study shows the individual variation in IgG to be less variable among captive koalas where IgG is measured over more timepoints after vaccination. Future studies should consider examining both the average cohort effect and the individual variability of immune responses to vaccination.

Keywords: adjusted repeatability, adjuvant, cohort, individual, mixed effects model, MOMP

## 4.2 Introduction

A desirable property of vaccine administration is that individuals elicit a predictable immune response (e.g., a predictable raise in pathogen specific antibodies) with minimal variation among individuals. Researchers commonly evaluate the capacity of vaccines to confer predictable immune responses by examining the average immune response of replicate individuals in a group following vaccination (i.e. cohort effect; e.g., Abraham et al. 2019; Pal et al. 2017a; Pal et al. 2017b; Tifrea et al. 2020). Thus, the variability of individual immune responses is often derived from the confidence for an average cohort effect. A detectable immune response at the cohort level is an essential component of assessing vaccine effectiveness, but the variability of individual responses within the cohort is often considered less explicitly. Understanding individual variability of immune responses to vaccination is important for humans, domestic animals and wildlife, particularly in the context of background differences in immune history, genetics, environment and behaviour (Lewnard and Cobey 2018; Posteraro et al. 2014). Indeed, it is possible for vaccines to have cohort effects, but with highly variable individual responses (see Figure 1 conceptual diagram). This can present a therapeutic challenge for confidence of individual outcomes when administered by physicians and veterinarians. This reasoning also applies to other responses often examined in medicine, such as decrease in pathogen load following treatment (Dukers-Muijrers et al. 2013). Within an epidemiological context, an effective vaccination (either therapeutic or protective or both) in a population with low individual variability should have a high proportion of individuals with a desired response to vaccination, thus making it easier to control infection or disease. This, however, is also

affected by a number of other factors of disease dynamics including control effort and the propensity for a pathogen to elicit a superspreading event (Lloyd-Smith et al. 2005).

*Chlamydia* vaccine development is one exemplary field of research where trials are conducted with a focus on cohort level effects (Lizárraga et al. 2019). The aim for many *Chlamydia* vaccine studies is to develop a vaccine against one of two bacterial species: 1) *C. trachomatis*, a human pathogen, or 2) *C. pecorum*, a livestock and wildlife pathogen. Within the last decade, significant progress has been made to develop a vaccine for koalas against *C. pecorum*, a pathogen contributing to wild koala population declines in Queensland and New South Wales, Australia (Phillips et al. 2019; Quigley and Timms 2020). Indeed, there are several measures of vaccination success that have been investigated at the cohort level including the effects of vaccination on *Chlamydia*-related disease, chlamydial organism abundance, host anti-chlamydial antibody abundance (Immunoglobulin G and A; IgG and IgA, respectively), koala immune cell proliferation, and the expression of several key koala cytokines (particularly interferon γ and interleukin 17; Phillips et al. 2019). Systemic anti-*Chlamydia* IgG abundance is one long lasting (>6 months) measure of vaccine success that has been recorded in recent vaccine trials. In this study, the individual variability of koala chlamydial vaccines was analysed using one measure of vaccination success, systemic anti-*Chlamydia* IgG abundance.

**4.3 Methods**

This study focussed on three koala *Chlamydia* vaccine trials by Waugh et al. (2016), Desclozeaux et al. (2017a), and Nyari et al. (2018) reporting systemic IgG values for all vaccinated individuals. Importantly, all of these studies reported cohort effects after vaccination (Appendix 4.1). These studies also varied in vaccine type, adjuvant used,

whether koalas were free-ranging (infected or unknown infection status prior to vaccination) or captive (uninfected prior to vaccination), number of timepoints, data were collected after vaccination, and number of koalas. Variation in these study details is described in Figure 4.2. With particular regard to vaccine type, Waugh et al. (2016) measured systemic IgG abundance for free-ranging koalas vaccinated with recombinant major outer membrane protein (rMOMP) that had a *C. pecorum* prevalence of 54% at the time of vaccination. Desclozeaux et al. (2017a) measured anti-Pmp and anti-chlamydial elementary bodies (EB, genotype G) IgG abundance for recombinant Pmp (rPmp) vaccinated koalas, and separately anti-MOMP F, anti-MOMP G, and anti-chlamydial EB (genotype G) IgG abundance for MOMP vaccinated koalas that had a *C. pecorum* prevalence of 13% at the time of vaccination. Nyari et al. (2018) measured anti-MOMP A, anti-MOMP F, anti-MOMP G IgG abundance from all vaccinated animals (either rMOMP, or synthetic MOMP peptides, sMOMP) with no reported *C. pecorum* prevalence at the time of vaccination. Non-vaccinated koalas and vaccinated animals who had missing measurements were excluded from further analysis. Koalas without detectable anti-*Chlamydia* IgG at baseline were also excluded from further analysis as this method was unable to reliably estimate individual variability for these individuals. This means that individuals had antibodies for *Chlamydia* and were either infected or likely had recovered from an infection. It is unclear whether the healthy koalas studied by Nyari et al. (2018) that were born and bred in captivity (with no reported *Chlamydia* prevalence) had previous chlamydial infections. Lastly, a log transformation ($\log_{10}(x + 1)$) was used for all IgG measurements (ELISA end point titres) for analysis.

To estimate the individual variability of each vaccine trial, methodology was adapted from the behavioural ecology literature (McEvoy et al. 2015) to first estimate individual predictability over time, then converting this to individual variability. For each trial, a mixed-effects model was created where time (modelled as a continuous variable) was fitted as a fixed effect and individual as random intercepts in a Markov chain Monte Carlo general linear mixed model (see Appendix 4.1 for model coefficients). Model coefficients and variances were estimated using the MCMCglmm package (Hadfield 2010) with R statistical software (v3.6.2) (Team 2017). Models were fit using the default prior for 200,000 iterations thinned at an interval of 20 after a burn-in of 25,000 iterations (i.e. posterior distribution n = 8,750). After running the model, an assessment of model fit was made from the mixing of chains per Gelman and Hill (Gelman and Hill 2006). The residual and individual variances were extracted from the posterior distribution and a modified equation described by Dingemanse and Dochtermann ("adjusted repeatability"; Dingemanse and Dochtermann 2013) was used to estimate individual variability:

$$individual\ variability = 1 - \frac{V_{ind_0}}{(V_{ind_0} + V_{e_0})}$$

where $V_{ind_0}$ is the variance across individuals (i.e. between-individual variance), and $V_{e_0}$ is the residual error (i.e. within-individual variance). We averaged the model posterior distribution to obtain a mean individual predictability and calculated a 95% confidence interval (CI) using the highest posterior density from an inverse density function.

The average individual variability of systemic IgG from the 12 assays (from the three studies) were tested for differences among studies that used: 1) free-ranging vs. captive koalas, and 2) vaccine adjuvants using ISC vs. Tri-Adjuvant (PCEP, poly I:C, and IDR1002). Two additional

comparisons were made for koala IgG responses to: 1) rMOMP vs. rPmp vaccination in free-ranging animals (n=6 assays), and 2) rMOMP vs. sMOMP vaccination in healthy, captive animals (n=6 assays). Comparisons (using the mean individual variability of each study) were tested using a non-parametric Kruskal-Wallis rank sum test with a significance threshold of $p<0.05$. Finally, linear regressions (and F-tests with a significance threshold of $p<0.05$) were used to test changes in individual variability given: 1) the number of koalas for each assay and 2) the number of different timepoints IgG was collected after vaccination.

**4.4 Results and Discussion**

Figure 4.1 conceptually illustrates different scenarios for both a cohort effect and individual variability outcomes. Veterinarians and physicians may consider the individual variability to be the likelihood that any given individual will respond to a treatment. In this case, we know the original studies reported cohort increases in systemic IgG after vaccination (Appendix 4.1). We were interested in measuring the likelihood that any individual koala would have an increased systemic IgG abundance after vaccination as compared to pre-vaccination levels. In Figure 4.1, a trial may result in undetectable cohort effects and either high individual variability (Figure 4.1a) or low variability (Figure 4.1b) in antibody responses. Figure 4.1c and 4.1d show cohort effects with either high individual variability (Figure 4.1c) or low individual variability (Figure 4.1d, the most desirable scenario).

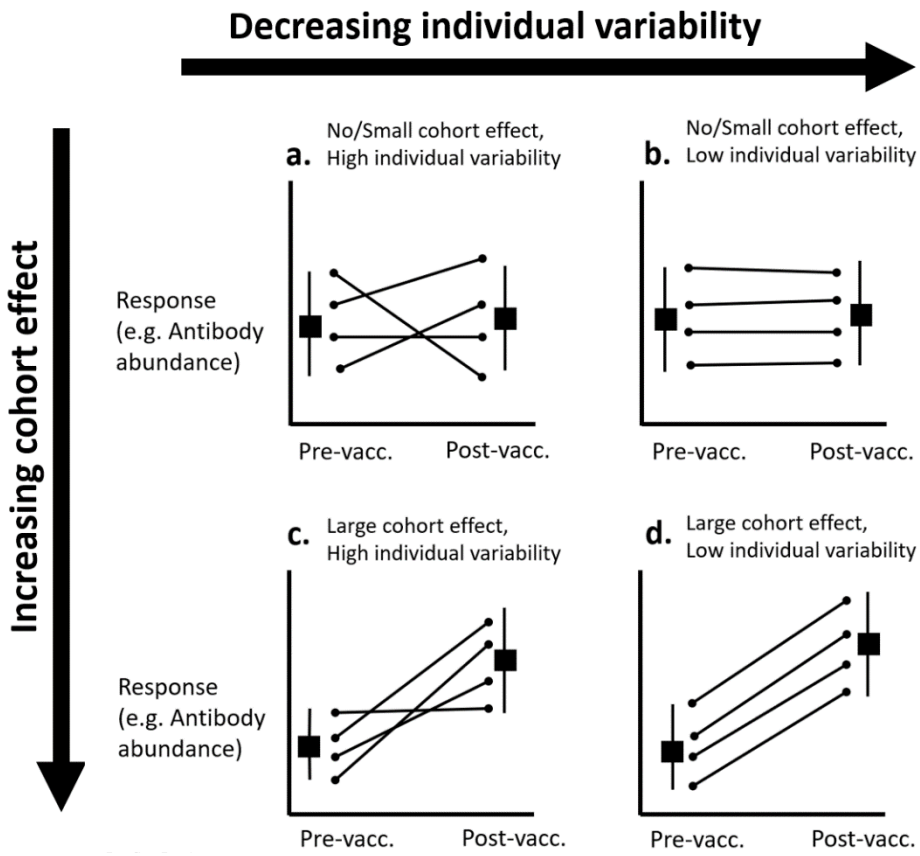


**Figure 4.1.** Conceptual diagram illustrating the relationship between cohort effects (squares with lines representing a mean ± variance) and individual variability (individuals are represented by two points connected by a line) of anti-*Chlamydia* host immune responses (e.g., antibody abundance or host cell cytokine expression) following vaccination. Scenarios include: no/small cohort effect and high individual variability (a), no/small cohort effect and low individual variability (b), large cohort effect and high individual variability (c), and a large cohort effect and high individual variability (d).

In Figure 4.2 we show the individual variability ($v$ ±95% CI) estimates from all three vaccine trials that show cohort effects for increased systemic IgG after vaccination (Appendix 4.1). Our results show individual variability reflective of Figure 4.1c and 4.1d. To evaluate the causes behind the patterns of variation in individual variability, we examined their relationship in the context of the four aforementioned comparisons. These results show that captive animals are likely to have lower variability in IgG responses after vaccination as compared to free-ranging koalas (Figure 4.3a). Specifically, the mean individual variability was lower for captive koalas reported by Nyari et al. (mean $v$ = 0.18; Nyari et al. 2018), relative to free-ranging koalas reported by Waugh et al. (2016) and Desclozeaux et al. (mean $v$ = 0.71; Desclozeaux et al. 2017a; see Figure 4.3a). Other than differences in veterinary care and diet, differences in individual factors such as age and genetic diversity are not disclosed in the original publications, thus making it difficult to extrapolate.

As stated in our methods, our analysis only included koalas with anti-IgG at baseline (i.e. no naïve individuals), suggesting that boosting the systemic IgG response of infected or previously infected koalas in the wild may be less predictable as compared to boosting the IgG response of captive animals despite these two populations have a differing infection prevalence. Vaccine adjuvants (for both captive and free-ranging individuals) included the tri-adjuvant (PCEP, poly I:C, and IDR 1002) or ISC. Our results show no clear pattern between individual variability between the two adjuvants (see Figure 4.3b). Within the trials using free-ranging koalas, only one of four measurements of systemic IgG had low individual variability after vaccination with rMOMP, and both measurements of IgG in animals vaccinated with rPmp showed high individual variability (Figure 4.3c). Thus, there was no clear pattern in the variation for koala IgG responses after vaccination with either a rMOMP

or rPmp vaccination ($p = 0.348$; see Figure 4.3c). Systemic IgG measurements from captive koalas (with detectable anti-*Chlamydia* IgG at the time of vaccination, but no reported chlamydial prevalence) collected by Nyari et al. (2018) had low individual variability and were comparable for animals vaccinated with either rMOMP or a vaccine consisting of synthetic peptides from a conserved region of MOMP (sMOMP; see Figures 4.3d and 4.4). This suggests that sMOMP may be equally as promising as rMOMP antigens in eliciting systemic IgG responses, warranting further investigating for use of sMOMP in future vaccination trials.

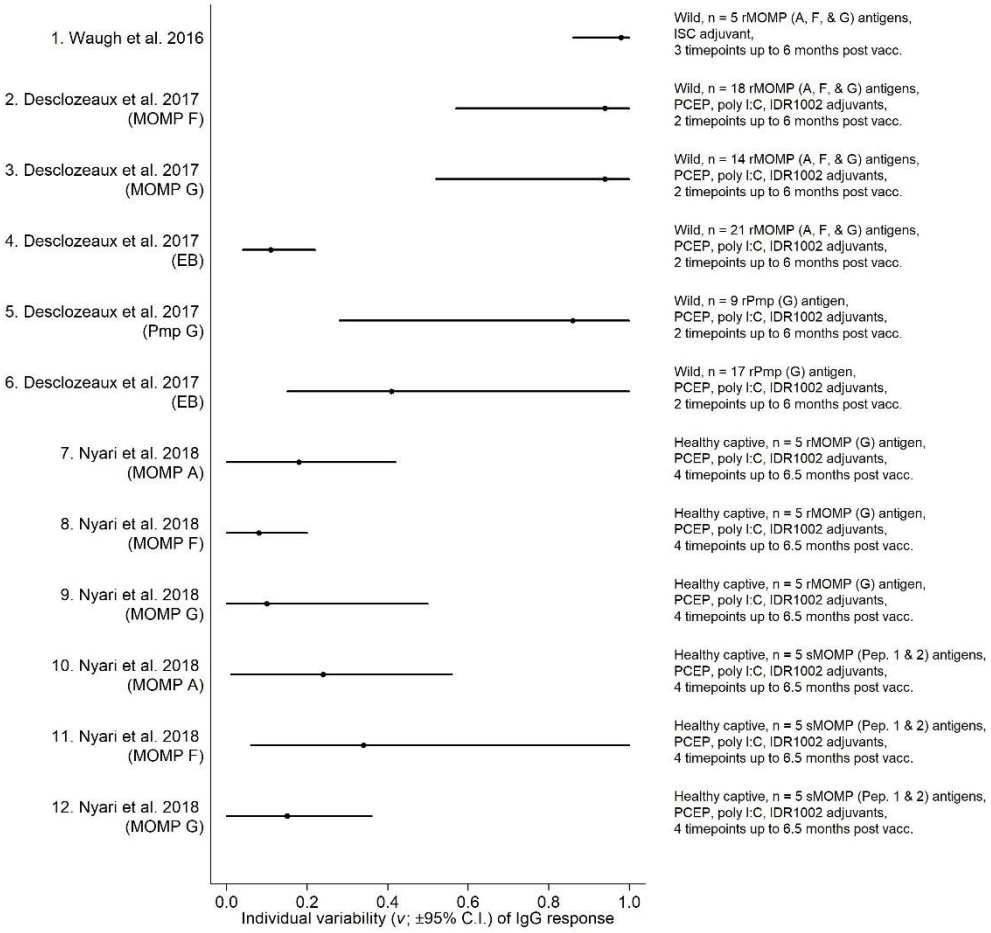


**Figure 4.2.** Individual variability as represented by *v* (± 95% CI) for a systemic IgG measurement in koalas in response to vaccination for three studies documenting cohort effects (see Figure 4.4 and Appendix 4.1). Multiple IgG targets were measured by Desclozeaux et al. 2017 and Nyari et al. 2018 and listed in parentheses below the study name. Detailed information is shown for each study to the right of the graph for the following: 1) healthy captive or free-ranging (wild) koalas, 2) the number of koalas with complete data in this analysis, 3) the vaccine antigen, 4) the vaccine adjuvant, and 5) the number of timepoints and maximum timepoint IgG measurements were collected.

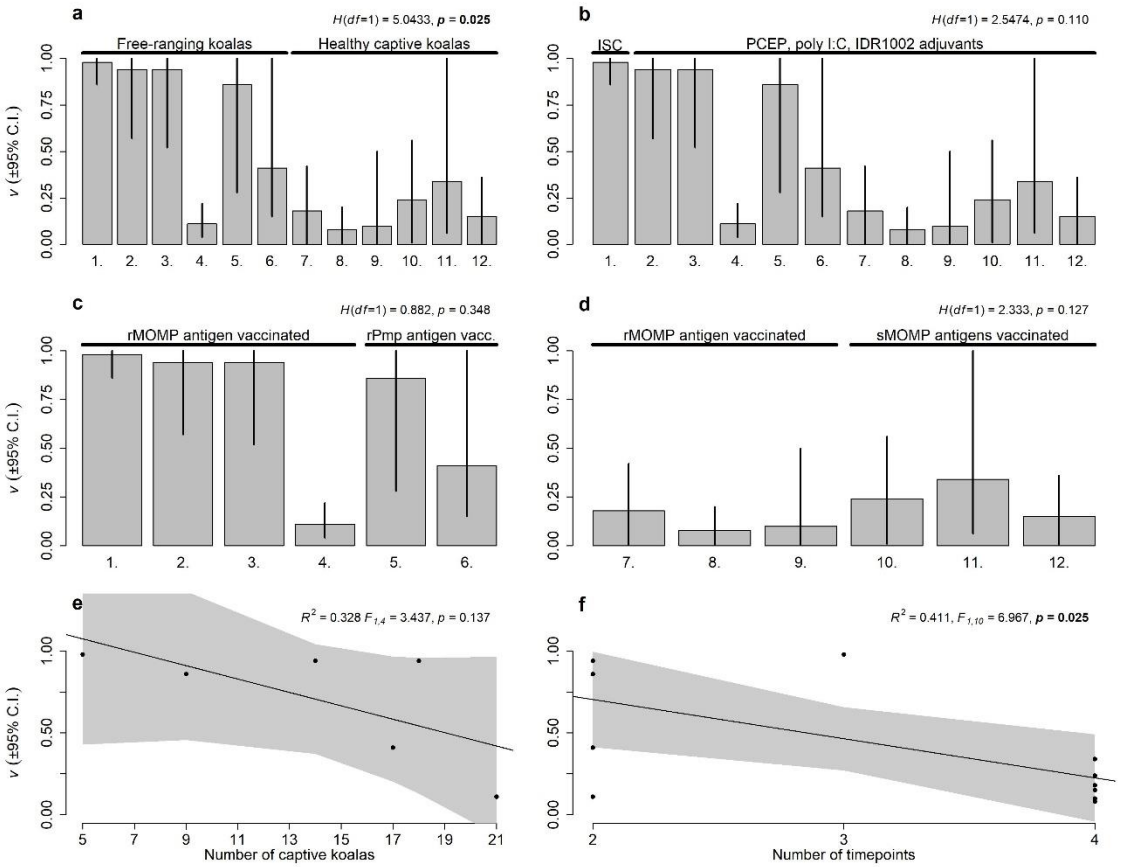


**Figure 4.3.** Individual variability (*v*; ± 95% CI) of systemic anti-*Chlamydia* IgG for free-ranging vs. captive koalas (a), adjuvant type (b), rMOMP vs. rPmp in free-ranging koalas (c), rMOMP vs. sMOMP in healthy captive koalas (d), number of captive koalas with complete data for each assay (e), and number of time points koala antibodies were measured (f). The results of a Kruskal Wallis rank sum test (a-d), and a regression (e and f) are shown at the top of each panel with p-values <0.05 in bold. Panels e and f show a solid line and grey area representing the linear regression and a 95%CI, respectively. Note: the number on the figure's x-axis corresponds to the study and assay described in more detail in Figure 4.2.

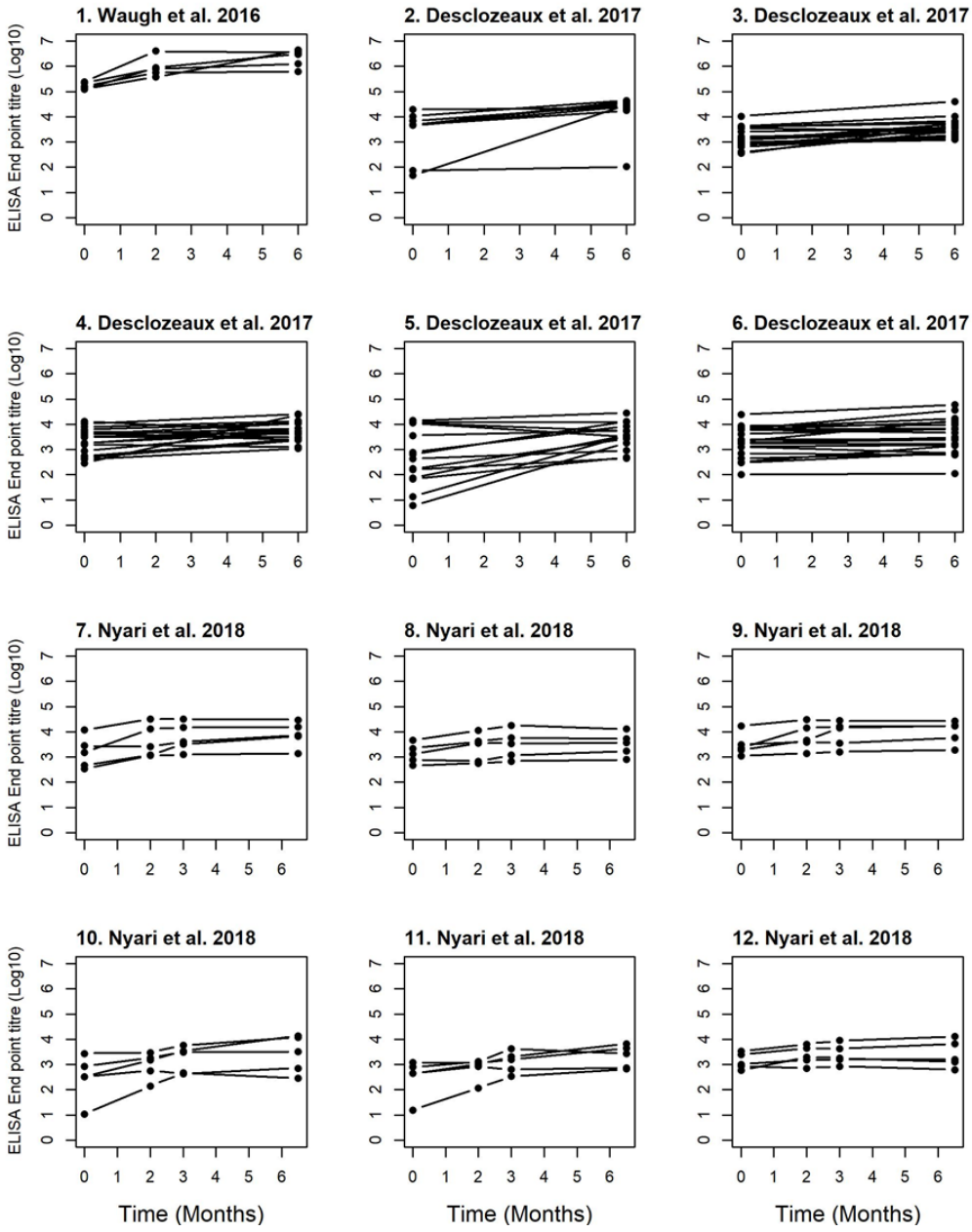


**Figure 4.4.** Individual $log_{10}$ transformed systemic IgG measurements collected from free-ranging animals by Waugh et al. (2016), Desclozeaux et al. (2017), and Nyari et al. (2018) up to 6.5 months post vaccination. Note: the number of each figure corresponds to the study and assay described in more detail in Figure 4.2.

Our analysis did not find an effect of the number of captive koalas on estimates of individual variability after vaccination ($F_{1,4} = 3.437$, $p = 0.137$; Figures 4.3e and 4.4). However, we did detect a relationship with the number of timepoints ($F_{1,10} = 0.776$, $p = 0.025$; Figures 4.3f and 4.4). Our results suggest that estimates of individual variability between four timepoints (studies 7 to 12) may be lower than if IgG measurements were taken only two or three times (studies 2 to 6 and 1, respectively). More research is needed to more clearly determine if this is an effect of sampling effort or is perhaps a confounding effect of differing laboratory methodologies. We note that the research on captive koalas with four timepoints were those of Nyari et al. (2018), so these results should be interpreted as reduced individual variability due to IgG measurements on captive koalas and/or over a greater number of timepoints.

In conclusion, recent trials of novel koala chlamydial vaccines, particularly those with captive koalas with more timepoints after vaccination, show lower individual variability in increasing systemic anti-*Chlamydia* IgG measurements. Though systemic IgG remains a common measurement of vaccination success, our recent meta-analysis shows IgA and IgG1 antibodies could also be considered as markers of a robust immune response against the abundance of chlamydial organisms (Lizárraga et al. 2019). The methods described here can be used to evaluate the individual variability of vaccines that already exist (e.g., attenuated *C. abortus* strain 1B; Longbottom et al. 2018) or novel vaccinations against pathogens of increasing importance (e.g., the zoonotic potential of *C. psittaci*; Jelocnik et al. 2019). We urge researchers to consider individual variability as an additional component of their analyses when evaluating cohort effects to treatment for humans or outbred animals.

Lastly, we recommend the reporting of individual responses to a given treatment over a study period as these data are valuable information in the effort to produce most desirable vaccine outcomes.

**Chapter 4 Supplementary Material**

**Appendix 4.1.** Model coefficients for each study's antibody assay. Note: the number of each row corresponds to the study and assay described in more detail in Figure 4.2.

| Study (see Fig. 2) | Assay target | Cohort effect (time as a fixed effect) | | | Individual (random effect) | | | Residual variance | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | 2.5%CI | 97.5%CI | Mean | 2.5%CI | 97.5%CI | Mean | 2.5%CI | 97.5%CI |
| 1. | IgG* | 0.168 | 0.088 | 0.088 | 0.005 | <0.001 | 0.021 | 0.148 | 0.053 | 0.284 |
| 2. | MOMP F | 0.059 | 0.008 | 0.107 | 0.015 | <0.001 | 0.320 | 0.199 | 0.089 | 0.320 |
| 3. | MOMP G | 0.136 | 0.020 | 0.240 | 0.055 | <0.001 | 0.383 | 0.787 | 0.279 | 1.304 |
| 4. | EB | 0.034 | 0.012 | 0.055 | 0.383 | 0.166 | 0.670 | 0.044 | 0.021 | 0.075 |
| 5. | Pmp G | 0.137 | <0.001 | 0.273 | 0.148 | <0.001 | 0.802 | 0.766 | 0.138 | 1.476 |
| 6. | EB | 0.066 | 0.036 | 0.093 | 0.100 | <0.001 | 0.214 | 0.064 | 0.019 | 0.148 |
| 7. | MOMP A | 0.103 | 0.053 | 0.152 | 0.575 | 0.035 | 1.706 | 0.069 | 0.026 | 0.128 |
| 8. | MOMP F | 0.053 | 0.027 | 0.081 | 0.489 | 0.046 | 1.351 | 0.021 | 0.008 | 0.039 |
| 9. | MOMP G | 0.073 | 0.028 | 0.117 | 0.380 | <0.001 | 1.115 | 0.057 | 0.021 | 0.108 |
| 10. | MOMP A | 0.135 | 0.070 | 0.202 | 0.764 | <0.001 | 2.232 | 0.128 | 0.043 | 0.241 |
| 11. | MOMP F | 0.125 | 0.064 | 0.186 | 0.375 | <0.001 | 1.124 | 0.106 | 0.032 | 0.240 |
| 12. | MOMP G | 0.038 | 0.004 | 0.068 | 0.310 | 0.024 | 0.913 | 0.029 | 0.011 | 0.055 |

*ELISA target not specified by Waugh et al. 2016.

## Chapter 5: Uncovering direct and indirect factors linked to repeat genital *Chlamydia trachomatis* infections: A focus on sexual practices

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Author's contributions: DL, BLQ, JSH, PT, and SC conceived and designed the research; JSH, LAV, WMH collected the data; DL performed the analysis; DL and SC interpreted results; DL, PT, and SC drafted the manuscript, and all authors participated in manuscript modifications.

This chapter changes the focus from host immune response(s) to infection and vaccination to investigating host sexual behaviour and subsequent chlamydial infection after antibiotic treatment. Despite the differences in this chapter as compared to the previous chapters, there remains a link to the overall thesis through quantitative analyses of these data at an epidemiological level where a chlamydial vaccine currently does not exist.

## 5.1 Abstract

**Background:** Repeat genital *Chlamydia trachomatis* infections remain problematic despite widespread access to antibiotics and screening programmes. Multiple factors are associated with repeat infections and these associations are often complex and not well understood. Both direct and indirect factors may affect the likelihood of repeat infections, but current methods to model these relationships are limited to direct relationships only. In our study, we used a novel approach to create and test causal statistical models to simultaneously identify both direct and indirect factors affecting repeat *Chlamydia* infection.

**Methods:** We used baseline data from questionnaires and a chlamydial genotype collected as part of the Australian Chlamydia Treatment Study. Individuals were grouped into either a repeat or non-repeat chlamydial infection group based on a PCR result at follow-up. Data were analysed using relative risks and Spearman's rank correlations and these results were used to create a hypothesized model. Structural equation modelling framework was used to first test the fit of the model to the data, and then to identify direct and indirect factors predictive of repeat chlamydial infection.

**Results:** Of the 239 women for which baseline questionnaire data were available, 33 had a positive *Chlamydia* PCR result following treatment, representing a 13.8% repeat positivity level. We found evidence that repeat chlamydial infections were indeed predicted by direct and indirect factors in our study. Repeat *C. trachomatis* infection was directly and positively predicted by one factor, inconsistent condom usage. We found that a number of factors predicted inconsistent condom usage and were indirectly associated with repeat infection; age, anal sex, sexual network size, and vaginal sex frequency.

**Conclusions:** We identified the direct and indirect factors associated with repeat *Chlamydia* infection. The indirect factors identified in our model highlight factors that may be missed using traditional statistical analyses, and nevertheless are important factors for healthcare providers to consider for controlling repeat chlamydial infections. The modelling approach described in this study can be used in epidemiological studies where multiple behavioural factors may affect a disease outcome.

Keywords: structural equation model; cohort study; inconsistent condom usage; azithromycin

## 5.2 Introduction

*Chlamydia trachomatis* infections are prevalent worldwide with the most recent estimates from the World Health Organization being between 127 and 131 million new infections per year (Organization 2016; Organization 2018). Untreated urogenital chlamydial infections are particularly problematic for women resulting in pelvic inflammatory disease (Price et al. 2016), and potentially ectopic pregnancy or tubal infertility (Reekie et al. 2019). Uncomplicated urogenital *C. trachomatis* infections are often treated with either azithromycin or doxycycline (Lau and Qureshi 2002). In some high-income earning populations approximately one in five women treated for an initial infection retested positive with *Chlamydia* within one year (Gaydos et al. 2008; Kampman et al. 2016; Rose et al. 2020). So, what factors are predictive of repeat chlamydial infections in treated women?

The factors strongly associated with retesting positive with chlamydial infections in women are complex and not well understood. Studies have shown a number of risk factors are associated with chlamydial infections in women such as (though not limited to) age (Forcey et al. 2014; Skjeldestad et al. 2009; Wilkinson et al. 2017), inconsistent condom usage (Forcey et al. 2014; Wilkinson et al. 2017), ethnicity (Chambers et al. 2018) and country of birth (Wilkinson et al. 2017), hormonal contraception use (Forcey et al. 2014), and number of male sexual partners (Forcey et al. 2014; Skjeldestad et al. 2009; Wilkinson et al. 2017). Indeed, there exist multiple genital chlamydial genotypes and some that may be related to persistent infections among some women (Suchland et al. 2017; Witkin et al. 2017). Reduced efficacy of antibiotics for rectal *Chlamydia* infections, particularly azithromycin, may lead to autoinoculation and repeat genital infections (Hathorn et al. 2012; Kong et al. 2016).

The number and complexity of causes of repeat infections is a problem that plagues the understanding of chlamydial biology and epidemiology, and this may be due to the indirect nature of some factors. The underlying reason for the difficulty behind understanding the epidemiological causes of repeat infection is that factors may be acting both directly and indirectly. However, as a field of research, chlamydial epidemiology has typically focused on direct factors using univariate approaches (e.g., crude odds ratios) often alongside more robust estimates from a multivariate framework (e.g., logistic regressions and adjusted odds ratios). Therefore, methods that consider direct and indirect factors may help advance our epidemiological understanding of repeat infections.

Structural equation modelling (SEM) is a valuable approach to tackle aspects of this intractably complex issue (Hanf et al. 2014). In recent years, SEM has been proposed as a useful statistical tool for epidemiology that extends beyond multiple logistic regression models by allowing researchers to test potentially confounding indirect factors within hypothesized models (Tu 2009). Specifically, SEMs utilize a framework of regressions between the hypothesized relationships that can be used to predict continuous or categorical (including binary) outcomes (Kupek 2006).

In this study, we aimed to apply SEM to a cohort of women treated for genital Chlamydia infection, as a novel approach to investigate factors, both indirect and direct, that are associated with repeat chlamydial infections. We used two univariate approaches to first analyse the baseline data, then used the results to guide the creation of a hypothesized model of direct and indirect factors associated with repeat *C. trachomatis* infection. We integrated this hypothesis in a SEM framework and tested its fit against our data to evaluate

if it was likely and then we identified the direct and indirect factors associated with repeat chlamydial infection.

## 5.3 Methods

The analyses performed in this study were designed around existing datasets for the Australian Chlamydia Treatment Study (ACTS) where the original study methodology has been published by Hocking et al. (2013) and briefly summarized in Appendix 5.1.

### *5.3.1 Outcomes and data collection*

The primary outcome for this analysis was genital *Chlamydia* repeat positivity detected by qPCR at least four weeks after treatment for genital *Chlamydia*. Thirteen questions from a baseline sexual practices questionnaire and one laboratory measurement (baseline chlamydial genotype) were analysed as factors affecting repeat chlamydial infection. A positive qPCR result after treatment could possibly indicate one or more of the following scenarios: 1) a new chlamydial infection obtained after azithromycin treatment from an infected partner, 2) a recurring chlamydial infection that previously entered a persistent form after azithromycin treatment, 3) failure of azithromycin treatment to clear an infection, or 4) autoinoculation from bacteria that reside in a different anatomical site (e.g. gastrointestinal tract). We understand that a positive PCR result for the chlamydial outer membrane protein A (ompA) gene (coding the major outer membrane protein, MOMP) could have multiple interpretations (Batteiger et al. 2010), but we chose to define repeat chlamydial infection as a positive Cobas 4800 CT/N result at least four weeks after treatment (i.e. baseline) to investigate if any factors are predictors of repeat chlamydial infections. All individuals with both a baseline questionnaire and a baseline laboratory result

along with at least one follow-up were included in the analysis. Individuals without a confirmed negative laboratory result after treatment were recorded as patients where treatment had potentially failed. Preliminary analyses where these patients were dropped from the analysis did not affect the results (Lizárraga et al. unpublished) and were included in the analysis due to the possibility of a repeat infection. For each questionnaire answer or laboratory result, a percentage of patients from either the repeat infection (n=33) or non-repeat infection (n=206) subgroups with that factor was calculated. These percentages were used to calculate a crude relative risk ($\%_{reinfected}$ divided by $\%_{non\text{-}reinfected}$). For interpretation, relative risks >1.5 and <0.5 were factors that increased or decreased the risk of being reinfected, respectively. If a measured variable was continuous, a mean ± 95% Confidence Interval (CI) was calculated (see Table 5.1 and Appendix 5.2).

**Table 5.1.** Baseline factors and number of patients that did or did not have a repeat *C. trachomatis* infection by the end of the study. Displayed are either number of patients (% of cohort) or means ± 95% CI. Relative Risk is given as the ratio of individuals within the repeat infection group (as a percentage) to the non-repeat infection group (as a percentage) for each baseline factor.

| Baseline factors | | Repeat infection* (n=33) | Non-repeat infection (n=206) | Relative Risk |
|---|---|---|---|---|
| Country of Birth: | Oceania | 12 (36.3%) | 72 (35.0%) | 1.0 |
| | NW Europe | 14 (42.4%) | 88 (42.7%) | 1.0 |
| | SE Europe | 0 | 2 (1.0%) | -- |
| | NE Asia | 3 (9.1%) | 12 (5.8%) | 1.6 |
| | SE Asia | 2 (6.0%) | 8 (3.9%) | 1.5 |
| | Americas | 1 (3.0%) | 17 (8.3%) | 0.4 |
| | Sub-Saharan Africa | 0 | 3 (1.5%) | -- |
| Age | | 24.0 ± 0.8 | 23.8 ± 0.2 | -- |
| Hormonal Contraceptive Use | | 20 (60.6%) | 132 (64.1%) | 0.9 |
| Estimated day in a 28-day cycle | | 12.9 ± 1.4 | 16.4 ± 1.0 | -- |
| The patient reported any anal sex | | 5 (15.2%) | 39 (18.9%) | 0.8 |
| The patient did not report using condoms | | 17 (51.5%) | 74 (35.9%) | 1.4 |
| Number of days since sex without a condom | | 12.4 ± 1.4 | 32.7 ± 12.5 | -- |
| Of the last three partners: number of partners the patient report never using condoms with | | | | |
| | 0 | 4 (12.1%) | 77 (37.4%) | 0.3 |
| | 1 | 14 (42.4%) | 70 (34.0%) | 1.2 |
| | 2 | 10 (30.3%) | 42 (20.4%) | 1.5 |
| | 3 | 5 (15.2%) | 17 (8.3%) | 1.8 |
| Number of partners the patient had in the last 3 months | | 2.7 ± 0.3 | 2.4 ± 0.1 | -- |
| Number of new partners the patient had in the last 3 months | | 2.2 ± 0.3 | 1.8 ± 0.1 | -- |
| Chlamydial ompA genotype: | d | 4 (12.1%) | 36 (17.5%) | 0.7 |
| | e | 19 (57.6%) | 91 (44.2%) | 1.3 |
| | f | 5 (15.2%) | 28 (13.6%) | 1.1 |
| | g | 2 (6.1%) | 23 (11.2%) | 0.5 |
| | h | 0 | 4 (1.9%) | -- |
| | i | 0 | 2 (0.5%) | -- |
| | j | 0 | 14 (6.8%) | -- |

| | | | |
|---|---|---|---|
| k | 3 (9.1%) | 9 (4.4%) | 2.1 |
| Previous *C. trachomatis* diagnosis | 10 (30.3%) | 46 (22.3%) | 1.4 |
| Previous PID diagnosis | 0 | 4 (1.9%) | -- |

*Reinfection determined by a positive qPCR result from vaginal swabs collected 28 to 56 days after treatment of an initial chlamydial infection with azithromycin.

Data were transformed to fit a 0 to 1 scale as described in Appendix 5.2. A Spearman's rank correlation was calculated for each factor to guide the creation of a global model to be tested. Factors that had a direct correlation with repeat chlamydial infection were included as direct factors of repeat chlamydial infection in the model. Any correlations with the direct factors just described were included as indirect factors (to repeat chlamydial infection) in the hypothesized model. A correlation with a p-value threshold of <0.05 was used.

Model fit was tested using three indices and a model was only accepted if it fit these indices: $\chi^2$ p-value >0.05, a comparative fit index >0.9, and root mean square error of approximation <0.05 (Kline 2015). Model pathways were estimated using a diagonal weighted least squares (DWLS) estimator for binary data and statistically significant model paths were determined for each DWLS p-value <0.05. All statistical analyses were conducted using R statistical software (v3.6.2) (Team 2017) and two packages in R: *lavaan* for structural equation models (Rosseel 2012) and *corrplot* for the creation of the correlation matrix (Zheng et al. 2006).

**5.4 Results**

Of the 239 women with complete data, 33 had repeat chlamydial infections during the trial. Of the 33 patients, two patients had a positive Cobas PCR result for MOMP after baseline (both had a follow-up 28 days after treatment) without a confirmed negative PCR follow-up result between the two positive results (i.e. only two measurements were taken at 56 days, both were PCR positive), potentially indicative of treatment failure. Of the 33 patients, 10, 11, or 12 had a positive qPCR result at 28, 42, or 56 days after treatment, respectively.

While the study was conducted in Australia, participants were originally from a range of countries (see Table 5.1). Two geographic areas were associated with a higher risk of repeat

infection as determined by country of birth data, northeast Asia and southeast Asia (crude RR of 1.6 and 1.5, respectively). Though observational, patients from the Americas had a lower risk for repeat chlamydial infection (crude RR of 0.4). The number of partners the patient reported 'never' using condoms with had an increasing relative risk with each level of this factor (crude RR of 0.3, 1.2, 1.5, and 1.8 for 0, 1, 2, 3 partners, respectively). Additionally, chlamydial genotypes g and k had a lower (crude RR of 0.5) or higher (crude RR of 2.1) relative risk for repeat infections, respectively. There were no clear relative risks (crude RRs were between 0.5 and 1.5) among patients with repeat infections and factors relating to hormonal contraceptive use, anal sex, or previous diagnoses (either *C. trachomatis* or PID).

We evaluated the interrelationships among all variables examined in this study (Figure 5.1). Only one baseline factor was directly correlated with repeat chlamydial infection: number of partners the patient reported 'never' using condoms with (Spearman's $\rho = 0.18$, $p = 0.006$, see Figure 5.1). We included the other two inconsistent condom usage factors as indirect variables as all three factors were positively correlated with each other: 1) time since last sex without a condom and 2) no condom usage indicated as a form of contraception (see Figure 5.1). Eight baseline factors were correlated with one or more of the inconsistent condom usage factors and these were included in the model as a factor indirectly affecting repeat chlamydial infection: 1) a northeast Asia country of birth, 2) age, 3) hormonal contraceptive use, 4) anal sex participation, 5) number of male sexual partners in the last three months, 6) number of new male sexual partners in the last three months, 7) frequency of vaginal sex, and 8) a previous chlamydial diagnosis (see Figure 5.2a for the hypothesized structural equation model).

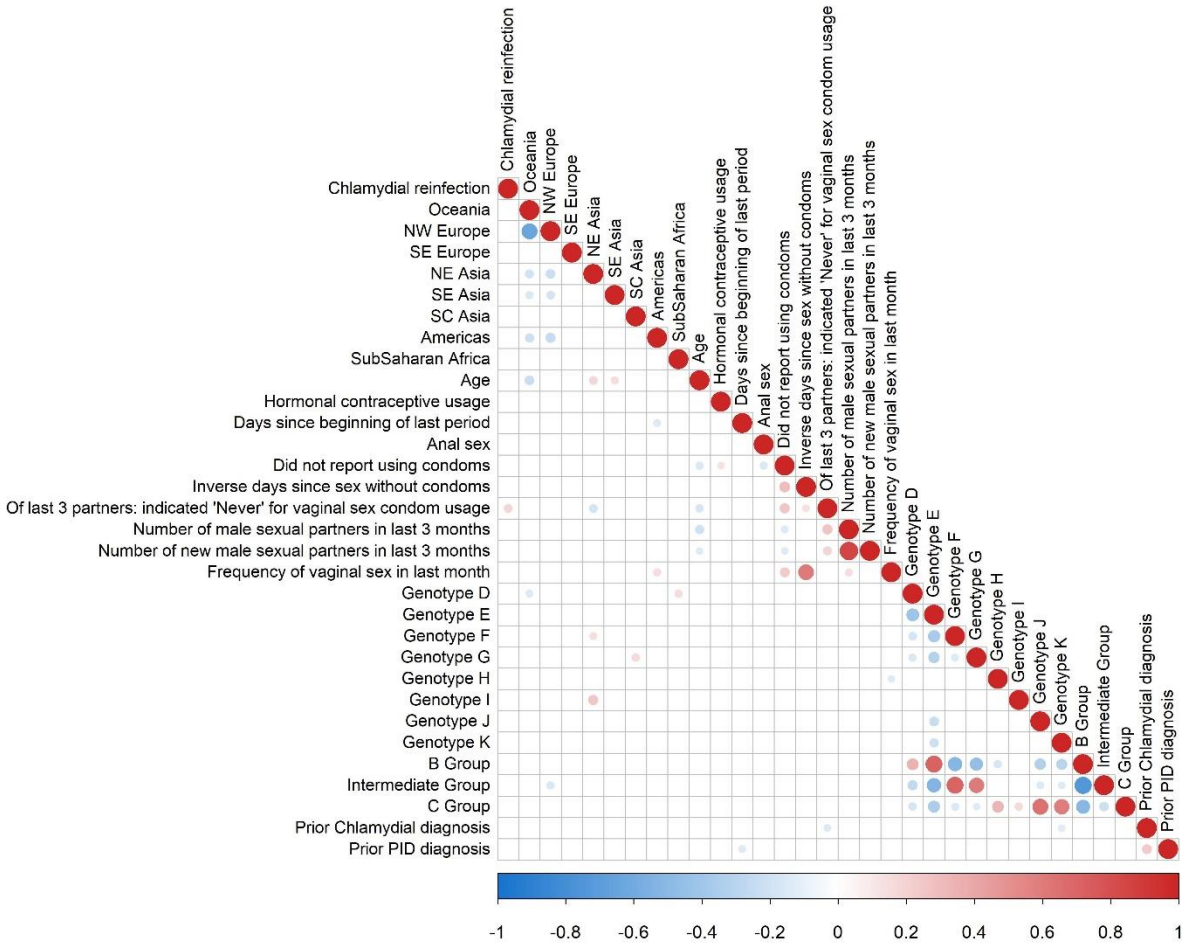
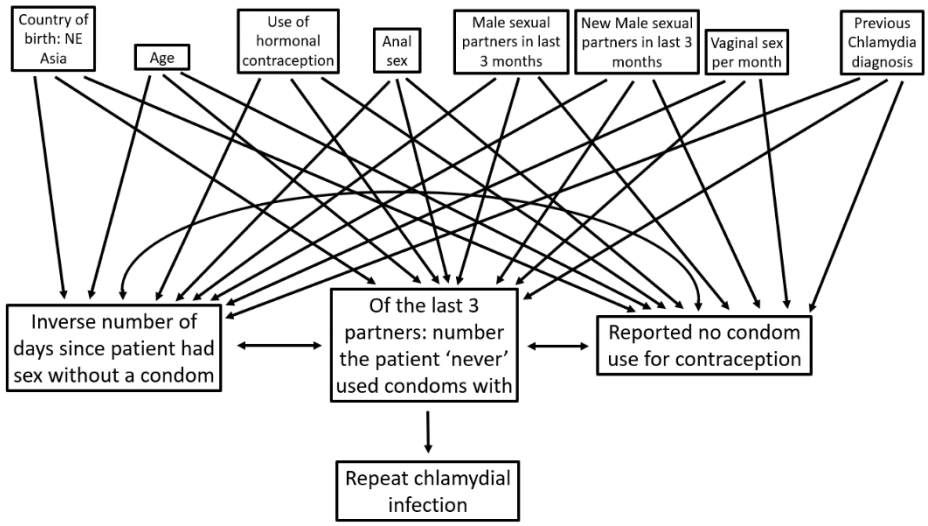



**Figure 5.1.** Correlation matrix using a Spearman's rank correlation of all factors listed in Table 5.1. The colour scale axis and the size of each dot indicates the strength of the relationship (Spearman's ρ), where blue and red colours are negative and positive relationships, respectively. Only correlations with a $p<0.05$ are shown.

**a**




**b**

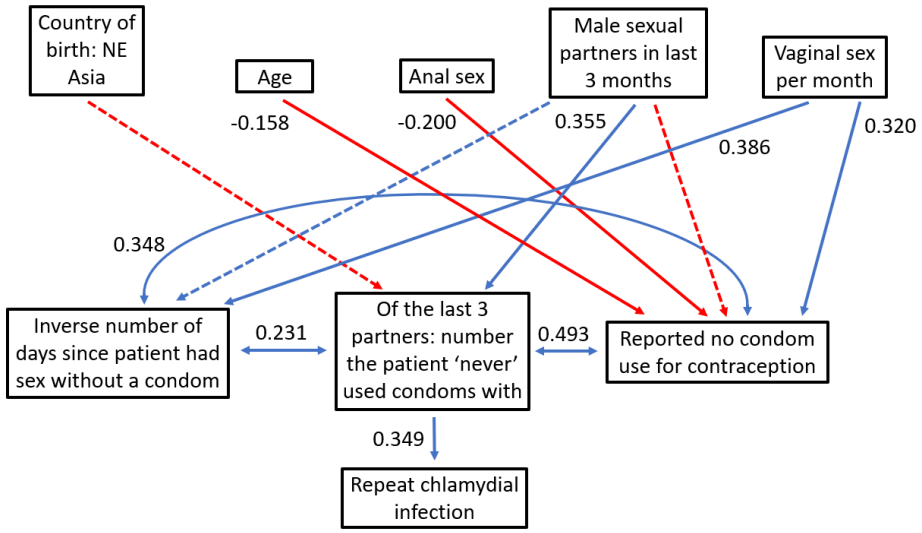



**Figure 5.2**. The global structural equation model that was informed as a result of the correlation matrix (see Figure 5.1) before being tested (**a**) and after showing the results of the tested model (**b**). Data used in these models were transformed based on transformations described in Appendix 5.1. Dashed arrows represent trending relationships with a p value between 0.1 and 0.05, while solid lines indicate a relationship with a p value of <0.05. Blue and red lines represent positive and negative relationships, respectively, and the numbers next to each line indicate the strength of the relationships standardized to all relationships in the model.

The hypothesized global model contained 12 factors with one factor directly predicting repeat chlamydial infection (see Figure 5.2b). The hypothesized global model was accepted as it fit three robust model fitting criteria ($\chi^2$ = 0.393, comparative fit index = 0.992 and root mean square error of approximation = 0.015). Agreeing with the relative risk and correlation results, the global model results show the number of partners a patient reported 'never' using condoms with as a positive predictor of repeat chlamydial infection (standardized estimate = 0.348, p<0.001). Figure 5.3 shows the raw data and the higher proportion of patients in the repeat infection group reporting partners they 'never' use condoms with (for all categories except for '0') as compared to the non-repeat infection subgroup, thus supporting this finding. Not surprisingly, all three inconsistent condom factors shared positive covariances (see Figure 5.2b for all three comparisons). The number of male sexual partners in a patient's sexual network was a positive predictor of how many partners the patient was likely to report 'never' using condoms with (standardized estimate = 0.355, p = 0.023). Upon investigating the raw data, this pattern can be seen (see Figure 5.4a). Frequency of vaginal sex per month (standardized estimate = 0.320, p<0.001), patient age (standardized estimate -0.158, p = 0.048), and participation in anal sex (standardized estimate = -0.200, p = 0.031) were predictors of a patient not reporting condom use as contraception (see Figure 5.4b, 5.4c, and 5.4d). Lastly, the frequency of vaginal sex per month was a positive predictor (standardized estimate = 0.386, p<0.001) for the inverse number of days since sex without a condom (i.e. 0 days since sex without a condom represents a maximum value for inconsistent condom usage) and investigating the raw data showed this pattern (see Figure 5.4e).

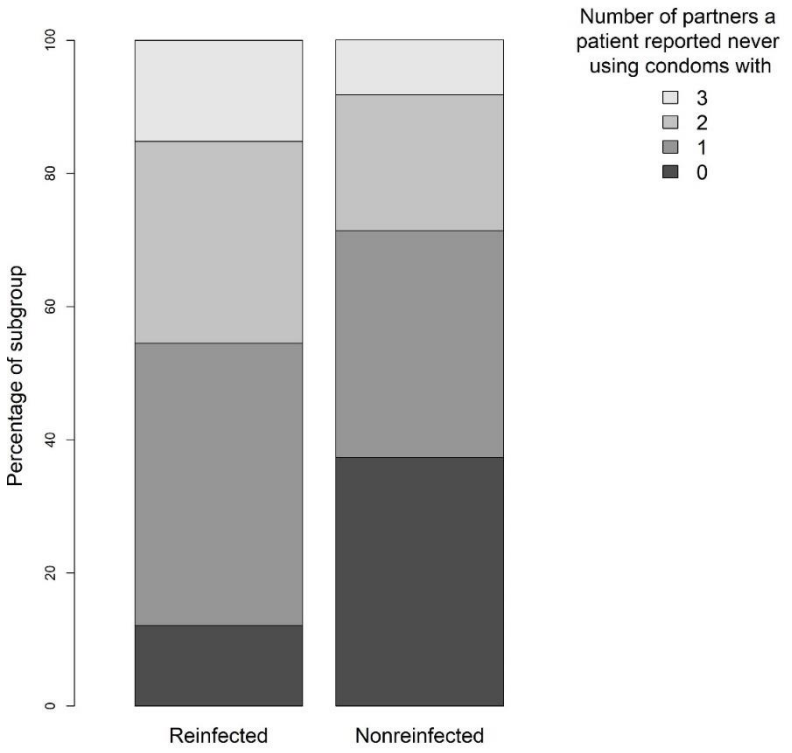



**Figure 5.3.** The percentage of the number of partners a patient reported never using condoms with for each of the reinfection and non-reinfection subgroups.

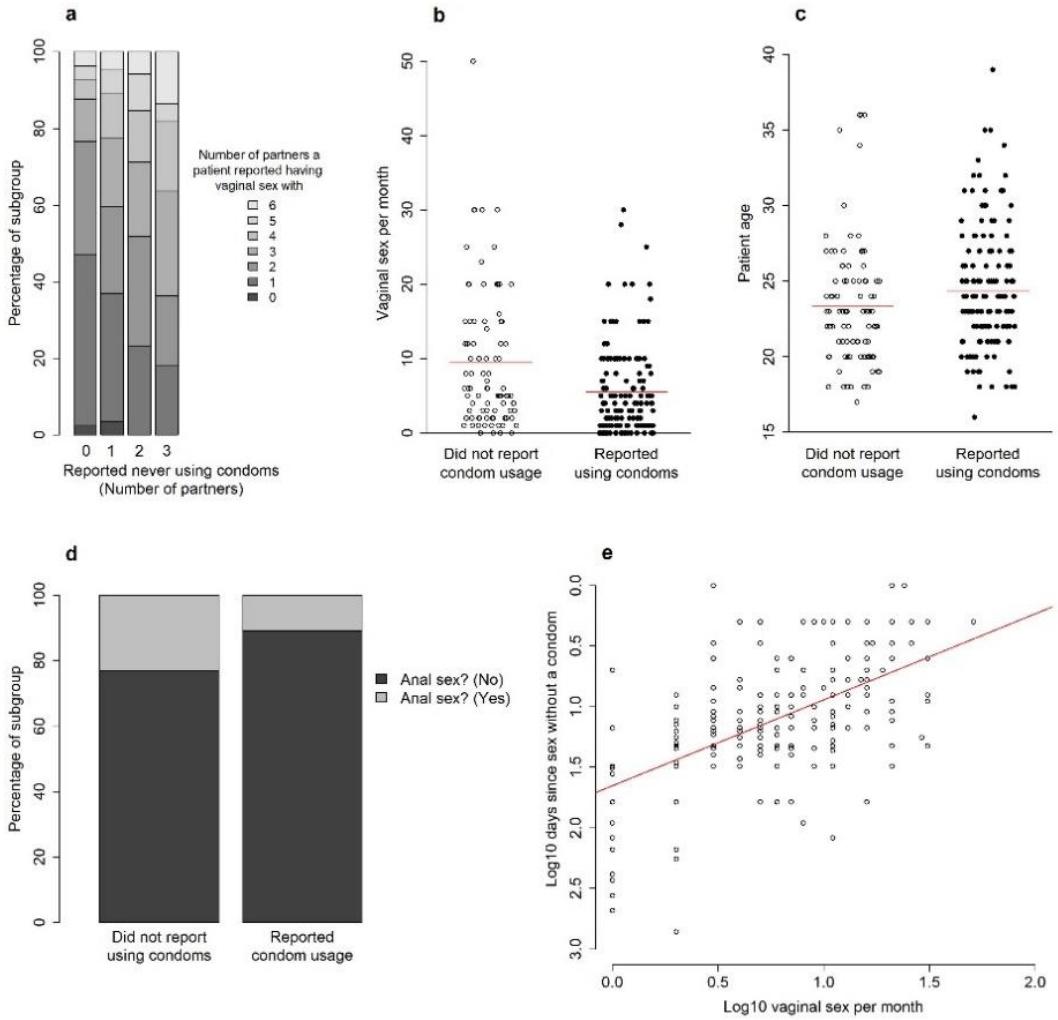



**Figure 5.4. (a)** Number of partners the patient reported never using condoms with (of the last three partners) and the number of male sexual partners in a patient's sexual network within the last 3 months (0 to ≥6) before azithromycin treatment (baseline). **(b)** Number of times a patient reported having vaginal sex per month and condom usage and **(c)** patient age and condom usage. Empty points represent patients that did not report using condoms and filled points are patients that reported using condoms before azithromycin treatment (baseline). Red bars represent the mean value for each condom usage category. **(d)** Condom usage and the percentage of patients that reported participating in anal sex. **(e)** Log transformed number of days since a patient has gone without using condoms and log transformed number of times a patient reported having vaginal sex per month showing a positive relationship (red line made from a regression). The y-axis is inverted to represent how the values were used in our structural equation model such that maximum values are representative of inconsistent condom usage (e.g. 0 days without using condoms are most inconsistent; see Appendix 5.1).

## 5.5 Discussion

In this study, we used structural equation modelling as a novel epidemiological approach to investigate the direct and indirect factors, particularly sexual practices, associated with repeat genital *C. trachomatis* infections after antibiotic treatment in a cohort of women in Australia. Of the baseline factors, inconsistent condom usage was directly predictive of repeat chlamydial infections. Additionally, patients that were younger, had a larger sexual network, reported a high frequency of vaginal sex per month, and those that did not report anal sex participation all were more likely to report inconsistent condom use for penile-vaginal sex. Each indirect factor either strengthens or weakens the relationship between inconsistent condom usage and repeat chlamydial infection, and identifying these complex interactions provides a unique insight for healthcare providers and clinicians to more effectively screen patients at risk for repeat infections. Furthermore, the approach in this study allows epidemiologists and researchers interested in complex study systems to create and test more meaningful mechanistic models given multiple risk factors.

Of the eight major factors that were measured pre-treatment, inconsistent condom usage was the only major factor directly predictive of repeat chlamydial infection. Specifically, evidence from both univariate methods and the final structural equation model support the association between number of sexual partners that a patient reported 'never' using condoms with and an increased likelihood of a repeat chlamydial infection. This association is consistent with previous findings that condom usage with male sexual partners (answered as either "usually, sometimes, never, or unsure", and compared to "always") is an associated risk factor for chlamydial infection (Forcey et al. 2014). This association is supported by several Bradford Hill criteria for causal inference, including temporality, a

dose-response (as evidence by the crude relative risks), plausibility, and consistency (Fedak et al. 2015). The structural equation model in this study highlights the importance of exposure as being critical for repeat infections, however, other major factors may be important and worth including in future models. These factors include both host genetics and the host immune response to infections that could not be measured in this study but may predispose some individuals to chlamydial infection and affect chlamydial disease severity (Agrawal et al. 2007; Mahdi 2002). Even as a component cause, transmission models show that improving condom usage (particularly among high risk groups) is one of the most important factors in decreasing the transmission of *Chlamydia* infections in women in Australia (Regan et al. 2008).

The wording of sexual behaviour questions is important for assessing patients potentially at risk for repeat infection during initial treatment. We found evidence for a direct relationship with repeat chlamydial infections from one out of three baseline questions regarding inconsistent condom usage. The direct relationship between condom usage (reported as a dichotomous question, with yes or no answers only) and repeat infections was not obtained in another cohort study of women in Australia by Walker et al. (2012). In this study the structure for sexual behaviour questions, particularly those regarding condom usage, was critical as not all condom usage questions were associated with repeat infections. Based on the findings of this study, clinicians and healthcare providers should ask patients to recall their condom usage with respect to three of the patient's most recent sexual partners as opposed to questions of contraception use with dichotomous outcomes.

Factors indirectly associated with repeat *Chlamydia* infections are of benefit for clinicians and healthcare providers seeking to better identify patients who may be at risk of

inconsistent condom usage. Based on the standardized estimates of our structural equation model, the larger size of a patient's sexual network and higher frequency at which they access their network are two important predictors of inconsistent condom usage behaviour. In our study, the estimates for these sexual network factors were greater in magnitude as compared to those of age and anal sex participation. A patient's sexual network and the frequency at which they access this network has been a positive predictor for chlamydial infection previously. Fortenberry et al. (Fortenberry et al. 1999) found that U.S. adolescents with ≥ 2 partners in the previous three months were more likely to have repeat infections (compared to patients with <2 partners) with a sexually transmitted infection after initial treatment with either *Chlamydia*, *Neisseria*, or *Trichomonas*. In another study of sexually active women in the U.S., having multiple sexual partners (>1), any new sexual partners, and inconsistent condom usage within three months were all predictors of repeat *C. trachomatis* infection (Burstein et al. 2001). A study in the U.K. of 16-24 women found that either two to three sexual partners in six months were predictors of repeat chlamydial infection (Lamontagne et al. 2007). A sensitivity analysis of screening in Australia estimated that two behavioural parameters were most important for chlamydia transmission at the population level: the frequency of sex acts for women between 20 and 24 years old, and the level of condom usage (Regan et al. 2008). In this study, age was not a direct factor, but rather an indirect factor negatively associated with inconsistent condom usage.

There are limitations associated with the approach used in this study. First, a multitude of different hypothesized models of a study system can be created. Arriving at the most meaningful model(s) has been a criticism of multiple model testing (Evans et al. 2012). To overcome this, we used a logical stepwise approach to guide the creation of the most

meaningful model and tested it to identify both direct and indirect factors linked to repeat *Chlamydia* infections. The methodology described in this study allows for the prediction of binary outcomes (repeat infection or not), which was previously thought to be a limitation of structural equation modelling (Der 2002). Second, only some demographic data were collected during the trial, so controlling for potentially confounding demographic factors (such as socioeconomic status) was not possible. Additionally, there are likely other potentially unmeasured confounding or contributing factors. Third, each of the sexual behaviour questions answered in ACTS were subject to recall bias. Lastly, our inability to determine chlamydial genotypes from follow-up samples due to low organism load limited identification of chlamydial persistence as measured by chlamydial ompA genotyping. Without more specific laboratory analyses it is difficult to determine whether a repeat infection is due to: 1) chlamydial persistence and resurgence after treatment, 2) a secondary persistent infection that remains latent until the primary infection has been cleared, 3) obtaining a new infection from an infected partner after treatment, or 4) autoinoculation from an infection at a separate anatomical site where treatment is less effective than in the genital tract (e.g., the gastrointestinal tract). The strength of chlamydial persistence is likely to be underestimated in our model, and it is important for future studies to characterize chlamydial strains in re-infected women.

**5.6 Conclusions**

While antibiotics are available to treat *Chlamydia*, repeat infections continue to be a problem. As the efficacy of azithromycin to treat chlamydial infections remains in question (Kong et al. 2014), many clinicians and healthcare providers may seek preventative strategies for controlling chlamydial infections. Identifying risky sexual practices linked to

repeat chlamydial infections is critical to focus prevention strategies on patients and their partners participating in such behaviours. Some behaviours may be different among women already displaying risky sexual practices resulting in an initial chlamydial infection as compared to women in the general population. In our study we identified inconsistent condom usage prior to baseline infection as a direct factor predictive of repeat *Chlamydia* infections. Age, number of male sexual partners, frequency of vaginal sex, and anal sex indirectly affected repeat infections through inconsistent condom usage. Many of these factors have been previously linked to repeat infections in the literature, though this is the first study to analyse them in a single, mechanistic model. The findings in this study highlight the need for contact tracing of sexual networks and continued education of condom usage when treating initial *Chlamydia* infections.

**Chapter 5 Supplementary Material**

**Appendix 5.1.**

The following are methods for data collection for the Australian Chlamydia Treatment Study described by Hocking et al. (2013).

*Study design and cohort recruitment*

An Australian cohort study design was used to estimate the proportion of women who retested positive with a genital chlamydial infection after azithromycin treatment for an initial genital chlamydial infection. Women 16 years of age or older were invited into this study after a genital C*hlamydia* diagnosis at either a clinic in Melbourne, Victoria or Sydney, New South Wales. Data was collected between November 2012 until December 2014. Participants were excluded for the following criterion: 1) having a current infection as part of a routine test, 2) having an associated infection with another bacterial sexually transmitted infection (STI), 3) having an associated pelvic inflammatory disease (PID) diagnosis, 4) reported the use of antibiotics two weeks before the diagnosis, 5) reported participating in commercial sex work, 6) women who do not wish to receive study packs, 7) women who do not have a mobile phone, 8) indicated a positive HIV status, 9) prescribed medication known to interact with azithromycin, and 10) have a known macrolide allergy. Clinical research nurses assisted with the collection of vaginal swabs and the completion of a sexual practices baseline questionnaire for each woman within the cohort. Clinic nurses also observed azithromycin treatment of the initial chlamydial infection for each of these women. Each individual in the cohort was asked to return to the clinic for sample collection one week after treatment and asked to complete follow-up questionnaires and collect vaginal swabs at home at 28, 42, and 56 days after treatment.

*Chlamydial quantification and genotyping*

An initial polymerase chain reaction (PCR) was performed on baseline and follow-up vaginal swabs using a COBAS 4800 CT/NG (Roche Diagnostics). Samples that had a positive cycle quantification (Cq) value $\leq$ 40.4 were defined as being positive for *Chlamydia*. For any positive samples, a second PCR was performed to determine the chlamydial genotype. The following primers based on the chlamydial major outer membrane protein (MOMP) were first used to get broad genotype groupings: 1) B group (genotypes B, D, E, L1, and L2), 2) intermediate group (genotypes F and G), or 3) C group (genotypes A, C, H, I, J, K, or L3). Then, a genotype specific PCR was used to determine the genotype(s) if possible. Samples with low concentrations of chlamydial DNA (whole sample Cqs between 36.5 and 40.4) resulted in a positive Cobas PCR where a genotype could not be specified. Quantification of chlamydial DNA for each PCR (qPCR) and background host beta-globin PCR measurements were taken, but not used in further analyses.

**Appendix 5.2.** Major factor from the literature, baseline questionnaire questions or laboratory tests, and transformations for each possible relationship to be tested in the global structural equation model.

| Major Factor | Baseline questionnaire question/laboratory test | Transformations for baseline questionnaire data or laboratory results |
|---|---|---|
| Host Ethnicity | What country was the patient born in? | 1 if from the following countries: Oceania, northwest Europe, southeast Europe, northeast Asia, southeast Asia, southcentral Asia Americas, Sub Saharan Africa |
| Age | What is the patient's age (years)? | Age first subtracted by 16 (the youngest) then divided by 36 (the oldest) |
| Hormones and hormonal contraceptives | Does the patient currently use oral contraception, Depoprovea, Nuvaring, Implanon, or the Mirena IUD? | 0 if no and 1 if any of the following: oral contraception (any), depoprovea, nuvaring, implanon, Mirena IUD |
| | When did your most recent period start? Are you not having periods currently? | Number of days from beginning of period to 28 days. 0 if period started the day of collection, 1 if days since beginning of last period was >=28 or if no period, assumed mid-luteal phase at 17.5 days. |
| Rectal infections | Did the patient report having anal sex with any number of partners? | 0 for no anal sex participation, 1 for participating in anal sex with any number of partners |
| Inconsistent condom usage | Does the patient currently use condoms as a form of contraception? | 0 for reporting using condoms, 1 for not reporting using condoms |
| | When was the last time the patient had sex without a condom? (days) | Scale from 0 (just had sex without a condom the same day as questionnaire answered) to 90 days. Any duration $\geq$ 90 days was treated as a 1 |
| | For the three most recent male sexual partners, how many would the patient report never using condoms with? | Number of partners listed as "Never" for condom usage divided by 3 |
| Sexual Network | How many new male sexual partners did the patient have in the last 3 months? | 1 if the number of partners $\geq$6, otherwise a 0 to 1 scale |
| | How many male sexual partners did the patient have in the last 3 months? | 1 if the number of partners $\geq$6, otherwise a 0 to 1 scale |
| | Number of times the patient had vaginal sex per month? | -Frequency of accessing sexual network (vaginal sex per month, >=30, 1; or 0 to 1 scale) |
| Chlamydial strain | Laboratory tested chlamydial genotype based on ompA | 1 for each of the following genotypes: d, e, f, g, h, i, j, k; 0 for all strains |

| | | |
|---|---|---|
| Previous chlamydial infection or PID Diagnosis | Did the patient have a previous C. trachomatis diagnosis? | 1 for yes, 0 for no |
| | Did the patient have a previous PID diagnosis? | 1 for yes, 0 for no |

# Chapter 6: General Discussion

## 6.1 Thesis overview

Globally, *Chlamydia* remains a major intracellular pathogen causing disease in a wide range of hosts including humans and wild koalas. Efforts to improve the control and epidemiological understanding of chlamydial infection and disease are increasing, and research that can support advances in these areas is critical. This thesis broadly sought to apply novel analytical methods to the field of chlamydial biology to test new hypotheses and advance the development of vaccines and control of disease. Within this thesis, there was a systematic review of the literature and a meta-analysis was performed to highlight the most effective chlamydial vaccine across a range of hosts and against different chlamydial species (Chapter Two). Structural equation models were created to understand the direct and indirect factors associated with vaccination success in free-ranging koalas (Chapter Three). Mixed effects models were used to evaluate the individual variability of koala systemic antibody response to chlamydial vaccination (Chapter Four). Lastly, as the previous chapters had broad applications to chlamydial infections and disease in humans, key direct and indirect factors associated with repeat chlamydial infections in a cohort of women in Australia were identified (Chapter Five).

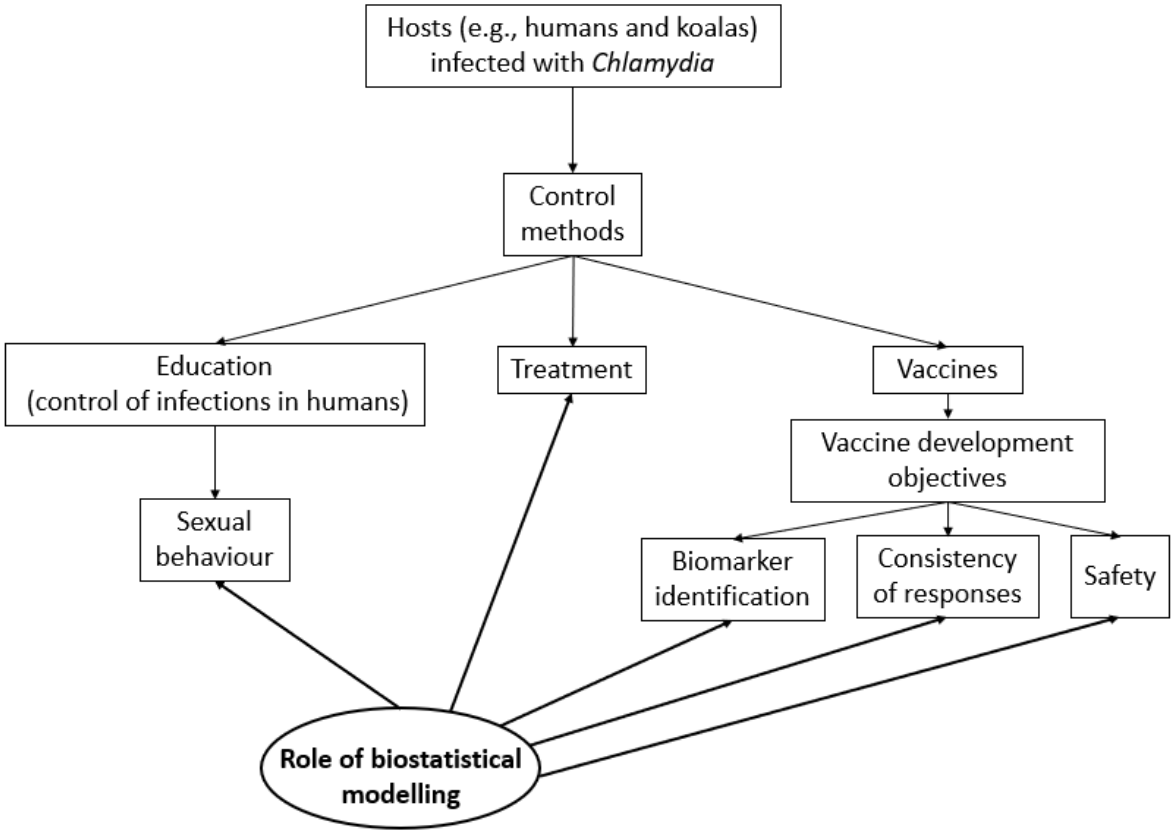



**Figure 6.1.** A conceptual diagram of the role of biostatistical modelling linked to a larger framework of *Chlamydia* control methods, including analytical links to sexual behaviour, treatment, and three vaccine development objectives: biomarker identification, consistency of responses, and safety.

## 6.2 Summary of key findings and their implications

Many researchers test different chlamydial vaccines and measure different immune responses as a measure of vaccination success (reviewed by Lizárraga et al. 2019; Phillips et al. 2019). The meta-analysis in this thesis (Chapter Two) of 165 different chlamydial vaccine studies shows that the majority of vaccine candidates identified among the literature against either *C. muridarum* or *C. trachomatis* using mice were effective in reducing chlamydial load. The majority of these studies reported two measures of success: 1) a change in chlamydial abundance (i.e. chlamydial load) and/or 2) a change in host immune parameter abundance (e.g., Badamchi-Zadeh et al. 2015). Chlamydial vaccines in mice most often increased the abundance of: IFNγ (interferon gamma), IgA (immunoglobulin A), IgG1, or IgG2a. Across the literature, an increase in host IgA and IgG1 abundance were most often correlated with chlamydial load decrease in vaccination trials. In all of these measures of vaccine success, there was a significant bias in the literature toward reporting measurements of chlamydial load reductions and host immune parameter increases after vaccination.

Studies focussing on developing a vaccine for koalas against *C. pecorum* typically only consider direct effects of vaccination outcomes when in reality immune and pathogen responses to vaccination are more complex (Desclozeaux et al. 2017a; Khan et al. 2014; Mathew et al. 2014; Nyari et al. 2018; Waugh et al. 2016; Waugh et al. 2015). The structural equation models in Chapter Three expanded upon previous studies. Specifically, models containing urogenital data showed that free-ranging koalas vaccinated with the major outer membrane protein (MOMP) against *Chlamydia* were more likely to have an increase in IL17

(interleukin 17) expression. Koalas with a lower urogenital chlamydial load were likely to have increased IL17 expression and were less likely to be diseased. There was no clear evidence that IL17 expression was linked to ocular or urogenital disease. Urogenital models containing IFNγ expression were less clear, possibly owing to difficulties in measuring this cytokine. Taken together, these results suggest that IL17 expression may be a more reliable marker of vaccination and koalas with a lower chlamydial load compared to IFNγ expression, despite the fact that previous literature (particularly studies using mice) often suggest that IFNγ expression is the most important measure of a robust immune response (Brunham and Rey-Ladino 2005).

Vaccination efforts are typically less understood at the individual level relative to average cohort effects in experimental trials (Fisher and Wakefield 2020; Kwong et al. 2010). Mixed effects modelling was used in Chapter Four to estimate the individual variability behind chlamydial infections in recent koala vaccine trial studies. These studies reported IgG responses as averaged (Desclozeaux et al. 2017a; Nyari et al. 2018; Waugh et al. 2016), cohort effects for vaccinated koalas comparing them to non-vaccinated control koalas, finding average increases in IgG in response to vaccination. The individual variability between changes in IgG responses for each study was evaluated using mixed effects modelling. The mixed effects models showed that there was more individual variation in measured antibody responses for free-ranging koalas that were also sampled less often after vaccination, as compared to the responses in a vaccine trial using captive koalas sampled more frequently.

Finally, the factors associated with repeat chlamydial infections in women are complex (Hocking et al. 2013), and direct factors associated with repeat infection have been

identified using logistic regression models. In Chapter Five, a structural equation model was created to test a number of behavioural factors associated with chlamydial reinfection using data from an epidemiological cohort study of women in Australia. This model showed inconsistent condom usage was directly linked to human repeat chlamydial infections while patient age, anal sex, and sexual network were indirectly linked to repeat chlamydial infections. This modelling approach expands upon traditional epidemiological approaches by showing which factors are indirectly associated with repeat infections (e.g., Forcey et al. 2014). The factors identified using this approach are useful for clinicians to improve screening practices for women at risk for repeat chlamydial infections.

The role of biostatistical modelling in this thesis is critical to providing new insights aimed at improving the control of *Chlamydia* infections (see Figure 6.1). A large part of my work focussed on data from trials aimed at vaccinating koalas, an animal host that is susceptible to chlamydial infection in the wild with a considerable number of parallels to human chlamydial infections. One critical aspect to creating a chlamydial vaccine for humans and koalas is the identification of import immune parameters associated with protection against chlamydial disease from a complex immune response (Brunham and Rey-Ladino 2005; Vasilevsky et al. 2014). The work in this thesis shows that an increase in anti-*Chlamydia* antibodies are often associated with chlamydial load decrease in hosts after vaccination as opposed to the expression of host immune cytokines, including IFNγ, a cytokine of major importance as indicated by the large number of studies reporting IFNγ expression measurements. The meta-analysis of *C. trachomatis* vaccine trials in mice (where many variables including host genetics and chlamydial infections are controlled) showed a trending link between IFNγ expression and chlamydial load decrease, though this

relationship was not seen among *C. muridarum* vaccine trials. In koalas, the relationship between vaccination and IFNγ expression was also unclear in my structural equation models (for either urogenital or ocular sites) six months after vaccination. These results bring into question the generalisability of IFNγ expression results in response to vaccination (Islam et al. 2018). In contrast, the meta-analysis in this thesis shows that increases in IgA and IgG1 antibodies were correlated with chlamydial load decrease, and systemic IgG measurements of koalas (both captive and free-ranging) also showed increases six months post vaccination compared to baseline measurements. This might suggest that antibody measurements are a more reliable marker for eliciting a protective immune response in naïve animals after vaccination as compared to measurements of cytokine expression. These results suggest that increasing an individual koala's anti-Chlamydia antibody abundance, is associated with decreases with chlamydial load. This result is not necessarily the elimination of an infection, but it does suggest that there would be a reduction in the shedding in these individuals, potentially affecting disease dynamics in a population. Additionally, the structural equation models in this thesis show that vaccination was not directly linked to chlamydial load decrease. This suggests that, at a population level, conservation efforts should be focussed on increasing anti-Chlamydia antibodies of uninfected individuals through vaccination.

Sampling frequency is important when comparing trials as some measured immune responses are short lived (e.g., IFNγ expression) and some are long lived (e.g., IgG and IgA; Stary et al. 2015). This has implications for comparing studies particularly laboratory mice versus domesticated livestock or free-ranging wildlife (Lizárraga et al. 2019; Phillips et al. 2019). It likely explains many differences among studies such as whether IFNγ expression is a useful indicator of protection or not. The work in this thesis identifies IgA and IgG,

particularly IgG1, as potential markers for successful chlamydial vaccines for humans. IL17 expression appears also an important koala cytokine as shown by structural equation modelling, though more research is needed to determine if IL17 expression is linked to protection against chlamydial infection.

### 6.3 Broader applications and future directions

This thesis describes the methodology for three approaches used to identify important outcomes to chlamydial treatment: meta-analysis effect size estimates, structural equation modelling, and individual variability estimates based on mixed effects modelling. These modelling approaches are less commonly considered within the field of chlamydial vaccinology, where simple univariate comparisons are commonly used to assess data. The modelling approaches I chose will continue to be important in future trials and can be used in fields outside of chlamydial vaccinology, particularly for the development of vaccines against other pathogens. For example, the novel coronavirus, SARS-CoV-2, has infected more than 10 million people globally (at the time of preparing this thesis) and is a pathogen of significant concern as it can lead to severe acute respiratory disease resulting in hospitalizations and in some cases, death (Wiersinga et al. 2020). Multiple international research groups are developing vaccines for SARS-CoV-2, with approximately 120 vaccine candidates in development. As more clinical trials are designed and the published results continues to grow, the methods described in this thesis can potentially be used to aid in: 1) identifying the most promising vaccine candidates from different published clinical trials, 2) evaluating direct and indirect factors associated with vaccine success, and 3) estimating the variability among vaccinated individuals. These modelling approaches may be particularly powerful as the complexity of the models may not be limited in sample size as clinical phase

II and phase III trials are designed around vaccinating hundreds to thousands of individuals, respectively.

The number of *Chlamydia* vaccination trials have steadily increased in recent years, making it critical to identify trends within this research and to focus research around vaccine development for the best possible success in disease control (Lizárraga et al. 2019; Phillips et al. 2019). Importantly, future meta-analyses aimed at identifying promising vaccines or factors associated with infection should consider disease as an important response to vaccination in addition to pathogen load. Other important vaccination factors should be a focus within meta-analytic models to further highlight important factors linked to vaccine success, including: adjuvant type, vaccination route, number of vaccinations, or site of sample collection. Models containing multiple moderators could also be used to test the effects of these aspects directly or indirectly using a combined meta-analytic structural equation model framework (MASEM; Jak and Cheung 2020). As chlamydial infections remain a significant pathogen to both koalas, humans, and other animals, future longitudinal studies will be critical to identifying factors linked to vaccination success and treatment failure. One complication encountered in this thesis was a constraint in model complexity associated with trials with small sample sizes. There exist alternative approaches to address this, such as the imputation of missing data (Royston 2004) or the inclusion of individuals with single measurements in individual variability models (Martin et al. 2011).

Finally, this thesis identified the importance of considering individual variation in addition to average cohort effects in response to vaccination. While analyses were focussed on koalas, there is a need to expand this to laboratory mouse, human, and domesticated animal

vaccine trials to better grapple with whether advances in vaccine development are producing more consistent host immune responses.

Collectively, the work in this thesis shows that the application of novel biostatistical modelling to the field of chlamydial biology offers unique insights into epidemiology and vaccinology, and that approaches used here can be applied to other infectious pathogens.

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